LOSS OF HETEROZYGOSITY ON CHROMOSOME 1 IN CERVICAL CANCER

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CONTENTS

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ABSTRACT

Background

An increasing number of studies indicate that tumourigenesis is related to abnormal cell growth promoted by mutation of proto-oncogene and tumour suppressor genes. Chromosome 1 anomalies are present in a high proportion of cervical carcinomas. Several types of structural change were repeatedly seen, the aberrations which included were deletions, isochromosomes, translocations and duplication. Frequent rearrangements of chromosome 1 were reported in cervical carcinomas by cytogenetic analysis. Allelic deletion at the locus on one chromosome leads to loss of heterozygosity (LOH) for the polymorphism. Chromosomal regions that are frequently lost are thought to harbour putative tumour suppressor genes.

Aims

1) To find a starting point for positional cloning of candidate tumour suppressor genes related to cervical cancer.
2) To open new strategies for predicting the prognosis of cervical cancer.

Methods

A PCR based method was used to detect LOH on chromosome 1 in 100 patients. Tumour tissues and blood samples from 100 cases of cervical cancer were collected. Twenty nine loci containing microsatellite markers and two variable number of tandem

repeats loci located on chromosome 1 were used in this study. The markers were labelled by [α-32p]ATP and PCR-amplified. The products were electrophoresed on 8% urea-formanide-denaturing-acrylamide gels and visualised by autoradiography. The data was statistically analysed using Pearson's chi-square test and Fisher's exact test.

Results

LOH was detected in 96 of 100 (96%) cases of cervical cancer. Thirty one to forty five percents LOH was found at the loci D1S2829 (1p31), D1S76 (1p36-35), D1S80 (1p36-35), D1S2663 (1p36.3) and D1S2757 (1q25). Twelve loci were involved in LOH in 20-28% of the informative tumours. All other loci had LOH in 20% or less of the informative cases. There is no difference in LOH in different age groups. The incidences of LOH were different between early- and late-stage at the loci D1S2829, D1S2757, D1S2647 and D1S2663 ($p < 0.01$-0.05). High grade tumour also shows significant increase in incidence of LOH at D1S2878 ($p < 0.05$). The incidences of LOH at the loci of D1S2829, D1S2757, D1S2703, D1S2635, D1S2647, D1S2845, D1S461, D1S2663 and D1S76 ($p < 0.01$-0.05) were significantly different among patients with different clinical outcomes.

Conclusion

Cervical cancer-specific tumour suppressor gene(s) may be located in chromosome 1p31, 1p36-35 and 1q25. LOH at certain loci is related to clinical stage, pathologic grade and clinical status. It may be as useful independent prognosticator(s).

摘 要

背景

越來越多的研究表明，腫瘤的形成與腫瘤基因和腫瘤抑制基因的改變所導致的細胞異常生長有關。

細胞遺傳學分析指出 1 號染色體的異常在子宮頸癌中占有較高的比率。經常可以見到幾種類型的結構改變，這些畸變包括缺失、單染色體、移位和復制。

染色體中一些位點上等位基因的缺失可導致其雜合性缺失(LOH) ，而這些丟失染色體的區域被認爲可能是特異的腫瘤抑制基因的所在處。

目的

用 PCR 方法檢測存在于宮頸癌 1 號染色體中的 LOH 目的於:
1 、提供進行克隆定位與子宮頸癌有關的腫瘤抑制基因的線索。
2 、找出新的判斷子宮頸癌預後的指標。

方法

收集一百例子宮頸癌病人的血液和腫瘤組織標本提取 DNA 。從 1 號染色體中選出二十九個微隨體標誌和二個短重覆標誌。這些寡核苷酸鏈標誌用〔α-32p〕ATP 標記和 PCR 擴增。 PCR 產物作凝膠電泳和放射自顯影。用 Pearson 氏卡方檢驗和 Fisher 氏精確檢驗法作數據的統計學分析。

結果

在 100 例子宮頸癌中共檢出 96 例患者存在有 LOH(96%)。在 D1S2829(1p31)， D1S76(1p36-35)， D1S80(1p36-35)， D1S2663(1p36.3)和 D1S2757(1q25)這 5 個位點上 LOH 的發生率爲 31%-45%。另外有 12 個位點上 LOH 的發生率爲 20%-28%。其餘位點上的 LOH 發生率爲≤20%。 LOH 的發生率與患者的年齡不相關。早期與晚期腫瘤患者在 D1S2829 、 D1S2757 、 D1S2647 和 D1S2663 ，這 4 個位點上 LOH 的發生率有明顯差別(P<0.01-0.05)。在低分化的腫瘤中 D1S2878 位點上的 LOH 發生率高於高分化的腫瘤(P<0.05) 。在 D1S2829 ， D1S2757 ， D1S2635 ， D1S2647 ， D1S2845 ， D1S461 ， D1S2663 與 D1S76 這 9 個位點上， LOH 的發生率與患者的臨床結果有顯著的關聯(P<0.01-0.05)。

結論

與子宮頸癌有關的腫瘤抑制基因可能位于染色體 1p31 、 1p36-35 和 1q25 。某些位點的 LOH 與腫瘤的臨床期別、細胞病理分化和臨床狀況有關聯，這些可能會成爲有用的獨立預測指標。

LIST OF ABBREVIATIONS

The following abbreviations are used in this thesis:

A adenine

ATP adenosine 5′ -triphosphate

bp base pair

C cytosine

dNTP deoxynucleoside triphosphate

FIGO The International Federation of Gynaecology and obstetrics

G guanine

h hour (s)

LOH loss of heterozygosity

M mole

Min minute (s)

mCi millicurie

mg milligram

ml millilitre

mmol millimole

ng nanogram

OCT optimal cutting temperature

PCR polymerase chain reaction

SDS sodium dodecyl sulfate

Sec second (s)

T thymine

U unit; uracil

V volume

WHO The World Health Organization

µg microgram

µl microlitre

Chapter One

Introduction

Cervical cancer remains one of the common female malignancies worldwide. Estimated 500,000 women are diagnosed with this malignancy each year. Associated mortality is exceeded only by that of breast cancer. In some countries such as Latin America, sub-Saharan Africa and southern and southeastern Asia, cervical cancer represents nearly all female malignancies (Herroro *et al.*, 1996). It continues to be an important cause of morbidity and mortality (Shingleton *et al.*, 1995). In Hong Kong cervical cancer ranked 4th for new cancers and 7th for cancer deaths in women. In 1993-1994, cervical cancer accounted for 6% of all newly diagnose cancer patients and 4% of all cancer deaths in females (Hospital Authority *et al.*, 1998).

It is clear that the discovery and characterisation of molecular markers of disease will be helpful for the prevention, early detection and effective treatment of cancers (Srivastava *et al.*, 1994).

Molecular studies of human cancer cells have revealed characteristic genetic alternations, usually consisting of deletions, translocations and gene amplification. The latter two abnormalities have been associated primarily with oncogene activation, while deletions are thought to indicate the location of putative cancer suppressor genes or antioncogenes, the loss or inactivation of which may play a role in tumour development

or progression (Fong *et al.*, 1989). Inactivation of tumour suppressor genes by mutations has been found in human tumour samples (Cox *et al.*, 1997), such as breast cancer, colon carcinoma, melanoma, pheochromocytoma, Wilms' tumour, neuroblastoma (Caron *et al.*, 1993), human male germ cell tumours (Mathew *et al.*, 1994), ovarian carcinoma (Abeln *et al.*, 1994), medullary thyroid carcinomas and phaeochromocytomas (Brodeur *et al.*, 1989). However, the molecular events underlying cervical cancer development are still unclear.

Chromosome 1 anomalies are present in a high proportion of cervical carcinomas. Frequent rearrangements of chromosome 1 were also reported in cervical carcinomas by cytogenetic analysis (Atkin and Baker *et al.*, 1982). Cytogenetic studies on 26 carcinomas of the cervix showed that chromosome 1 was consistently involved in the changes. Several types of structural change were repeatedly seen, the aberrations which included were deletions, isochromosomes, translocations and duplication (Atkin *et al.*, 1978).

In the tumour cells, mutation of a tumour suppressor gene is frequently accompanied by loss of the other non-mutated allele together with portions of adjacent DNA. This DNA loss may be detected using polymorphic markers for the affected locus and, where a marker is informative, deletion of the locus on one chromosome leads to loss of heterozygosity (LOH) for the polymorphism (Bethwaite *et al.*, 1995). Therefore, chromosomal regions that are frequently lost are thought to harbour putative tumour

suppressor genes (Rader *et al.*, 1996). Detection of LOH can provide a starting point for positional cloning of candidate tumour suppressor genes. Furthermore, it can open new strategies for predicting the prognosis.

Chromosome 1 changes are frequent in virtually all forms of human cancer. The existence of 1p deletions has been confirmed in a number of studies at frequencies ranging between 22% to 89%, with an average of about 30% of the tumours (Hunt and Tereba *et al.*, 1990; Peter *et al.*, 1992; Takayama *et al.*, 1992; Caron *et al.*, 1993; Schwab *et al.*, 1996). This would suggest an important role of chromosome 1 aberrations in tumourigenesis (Schwab *et al.*, 1996).

Loss of heterozygosity (LOH) has been identified at a number of loci in cervical cancer. Deletion mapping has shown loss at chromosome 3p13-p14.3, 3p14-p21 and11q23 (Bethwaite *et al.*, 1995), 2q, 4q, 5p, 6p22-21.3, 6q, 11q23.3, 11q13, 19q (Rader *et al.*, 1996) and 17p (Park *et al.*, 1995). A number of chromosome 1 studies of carcinoma of the cervix have been reported. One study has found frequent LOH at the D1S76 and D1S80 loci on chromosome 1p36 in 26 of 44 (59%) invasive cervical carcinomas (Wong *et al.*, 1993). Thus, the evidence of LOH on chromosome 1 in these cancers needs to be further evaluated with more sensitive methods, such as polymerase chain reaction (PCR).

Polymerase chain reaction (PCR) is a powerful technique to study the DNA. One of its applications is to identify the gene mutations. The method is based on the fact that the electrophoretic mobility of a DNA molecule through a polyacrylamide gel depends on the size and shape of the DNA molecule. DNA has a folded structure that is determined by intramolecular interaction related to its base sequence. Under denaturing conditions, the DNA unfolds. Double strand DNA splits into single strand DNA. Compared with the abnormal single strand, DNA has a different pattern in a polyacrylamide gel. Because of its sensitivity and efficiency, it has been widely applied in all areas of molecular biology and human genetics (John *et al.*, 1989).

Many deletions are believed to involve loss of tumour-suppressor genes, only a few of which have been cloned and sequenced (Bethwaite *et al.*, 1995). A cytogenetic study of carcinoma of cervix showed consistent changes in 4 chromosomes, namely 1, 3, 11, and 17 (Chung *et al.*, 1992). These chromosomal regions may contain oncogenes activated by rearrangements or tumour suppressor genes inactivated by deletion. This study looks into the mapping of potential tumour suppressor genes on chromosome 1 of cervical cancer.

The objectives of this study are as followings:

1) To find a starting point for positional cloning of candidate tumor suppressor genes related to cervical cancer.

2) To open new strategies for predicting the prognosis of cervical cancer.

Chapter Two

Literature Review

2.1 Epidemiology and aetiology of cervical cancer

2.1.1 Incidence and mortality

Cervical cancer is one of the major causes of cancer deaths on women world-wide. It leads to approximately 500,000 deaths annually in the world (Michael *et al.*, 1996). It is the second most common cancer among women, with an estimated 471,000 new cases diagnosed and 213,000 deaths occurring in 1990 (Pisani *et al.,* 1993; Rolando *et al.*, 1996*)*. In developing countries where disease prevalence is high, it remains the common cause of most cancer death in women .

The incidence and mortality from cervical cancer vary widely among countries, with up to 10-fold differences between high- and low-incidence areas. The areas with the highest incidence are Latin America, sub-Saharan Africa, and southern and southeastern Asia, while those with the lowest are Western Europe, North America, the Middle East, and China. In addition, regional variations within a country are very marked, with a higher rate in the rural and less developed areas (Herrero *et al.,* 1993; Rolando *et al.,* 1996).

The geographic distribution of cervical cancer is highly variable. The highest age-adjusted death rates are found in Central and South America and in Eastern Europe.

Mexico has the highest mortality rate, with 1,509 deaths per 100,000 population, Similarly high rates are observed in Chile, Costa Rica, Romania, Venezuela, and Poland. By contrast, the lowest rates are observed in Italy, Greece, Israel and Finland. The wide variation in incidence can partially be explained by the differences in the prevalence of risk factors for cervical cancer, inclusive of sexual practices, access to health care, and socio-economic status (Stephen *et al.*, 1996).

In Hong Kong cervical cancer ranked 4th for new cancers and 7th for cancer deaths in women. In 1993-1994 cervical cancer accounted for 6% of all newly diagnosed cases and 4% of all cancer deaths in females. There were 457 newly diagnosed cases in 1994. The median age at diagnosis and death was at 56 and 66 respectively (Hospital Authority *et al.*, 1998).

2.1.2 Aetiology

Of the established risk factors for the development of cervical cancer, those relating to female sexual behaviour are most consistently found and reported. Young age at first intercourse, multiple sexual partners, and high parity are cited as the major risk factors (Stephen *et al.*, 1996). Other risk factors such as smoking, nutrition and hormonal contraception may be secondary to human papilloma virus (HPV) and act by mechanisms that promote neoplastic changes.

The prevalence of cervical cancer was higher in married than in unmarried women and that the rates increased steadily between the ages of 30 and 60 years. The lowest rate of cervical cancer is among single persons and the highest rate is among parous and widowed persons. On the other hand, risk factors for patients with adenocarcinoma are different from that of squamous cell carcinoma. Patients with adenocarcinoma tend to be better educated and more affluent. They may have less sexually activity during their adolescent years. The ages are trend to less than 35 years (Shingleton *et al.*, 1995).

2.1.2.1 Oral contraceptive pills and cervical cancer

Recent well-controlled studies have revealed excess risk in women using oral contraceptive pills (OCP) for 5 or more years. Additionally, a twofold risk of high-grade cervical intraepithelial neoplasia (CIN) has been associated with six or more years of OCP use. A study in the United States showed that women using OCPs and not using barrier methods of contraception had a significantly increased risk of cervical cancer compared to controls, those using both methods had an insignificant increased risk. An increased incidence of adenocarcinoma of the cervix observed in young, middle- to upper class women spawned the hypothesis of an association between this particular cancer and OCP use (Shingleton *et al.*, 1995).

2.1.2.2 Human papilloma virus (HPV) and cervical cancer

The major risk factors predisposing to the development of invasive cervical cancer are strongly correlated to sexual behaviour. Numerous studies support the theory that

this cancer is a sexually transmitted disease and the causative agent is HPV (Münger *et al.*, 1995). Human papilloma virus has many strains and is a deoxyribonucleic acid (DNA) virus that cause benign and malignant epithelial hyperproliferation at selected body sites. HPV infections of the anogenital tract are the most prevalent sexually transmitted diseases of viral aetiology (Roland *et al.*, 1997). The finding of HPV DNA in up to 90% of squamous cell cervical cancers supports this theory. Epidemiological studies have shown that infection with a high-risk HPV is a significant risk factor for developing cervical neoplasia (Münger *et al* 1995). One multivariate analysis study has shown that infection with HPV types 16, 18, 31 and 33 are strongly associated with development of cervical cancer (Reeves *et al.*, 1989; Benjamin *et al.*, 1996; Roland *et al.*, 1997).

2.1.2.3 Immunity and cervical cancer

The patient's immunologic status plays an important role in the clinical expression of genital HPV infection and CIN. The evidence for an association between human immunodeficiency virus (HIV), HPV, and cervical cancer is compelling. These are sexually transmitted diseases that share common behavioural risk factors. It is known that HIV-seropositive women have increased rates of genital HPV infection and CIN. In one study, 70 percent of symptomatic HIV-positive women had genital HPV infection and more than 50 percent of them had abnormal cervical cytological results consistent with a squamous intraepithelial lesion. CIN and cervical cancer appear to be more

aggressive in HIV-positive women compared with HIV-negative women (Atkinson *et al.*, 1995).

2.1.2.4 Socio-economic differences and cervical cancer

Low socio-economic status has consistently been cited as being a risk factor in the development of cervical cancer, though it is difficult to evaluate as an independent variable. An association between cigarette smoking and the development of cervical dysplasia and invasive cervical cancer (ICC) has been reported in several previous studies (Stephen *et al.*, 1996).

2.1.2.5 Smoking and cervical cancer

The recent finding of an increased risk of squamous cell cervical cancer and not adenocarcinoma in smokers strengthened the preferential effect on squamous cell transformation (Stephen *et al.*, 1996). The effect of smoking that may have on lowering serum beta-carotene levels has been proposed as an indirect mechanism of carcinogenesis. High nicotine and cotinine levels have been found in cervical mucus, leading some biologically plausibility of carcinogenesis to the observed association. However, it is still unclear how or if these agents play a significant role in the genesis of cervical cancer.

2.1.2.6 Male role and cervical cancer

Sexual behaviour plays a role in the aetiology of cervical cancer. Wives of men with penile cancer had significantly elevated rates of cervical cancer (Graham *et al.*, 1980;

Peter *et al.*, 1969; Bornstein *et al.*, 1995). Kessler (Kessler *et al.*, 1977) followed men who remarried after their wife succumbed to cervical cancer and found the new wives to have a 2-fold increased rate of cervical cancer and a higher number of abnormal Papanicolaou smears compared with control women. Two studies showed that women with cervical cancer were five to seven times more likely to have a husband who had had ten or more sexual partners than were controls (Pridan *et al.*, 1971; Zunzunegui *et al.*, 1986).

2.1.2.7 Nutrition and cervical cancer

The relationship between developing cancer and dietary excesses and deficiencies is a contemporary medical topic of great interest to the general public. In a study of women in New York City, 3-day dietary records revealed a significantly lower intake of vitamin C in women with dysplasia compared to those without dysplasia. A follow-up study comparing blood levels of vitamins C confirmed significantly lower levels in patients with dysplasia compared to controls. Though recent studies have confirmed this association in patients with dysplasia, the data linking invasive cervical cancer (ICC) and dietary deficiency of vitamin C are conflicting (Stephen *et al.*, 1996).

2.2 Oncogene and tumour suppressor gene

2.2.1 Oncogene

In 1914, Boveri first suggested that an aberration in the genome might be responsible for the origins of cancer and many possible genetic mutations are involved in

the transition from a normal cell to a malignant one. Aberrant expression of proto-oncogene which is in control of normal cell growth and inactivation of tumour suppressor genes which normally act to control cell proliferation are associated with tumourigenesis (Macdfonald *et al.*, 1991). The mechanism of genetic damage includes point mutation, gene amplification and gene rearrangement (Kurzrock *et al.*, 1995).

Proto-oncogenes encode products that control cell growth and differentiation. When mutated, they may become oncogenes, which can cause cancer. Most oncogenes act as dominant mutations and cause cellular growth rates to increase. Retroviruses are capable of inserting oncogenes into the DNA of a host cell, thus transforming the host into a tumour-producing cell. The study of such retroviral transmission has identified a number of specific proto-oncogenes through their oncogenic variants (Jorde *et al.*, 1995).

Currently about 60 different oncogenes have been identified. In normal cells, the expression of these proto-oncogenes is tightly controlled and they are transcribed at the appropriate stages of growth and development of cells. However, alterations in these genes or their control sequence leads to inappropriate expression. Proto-oncogene is involved in the basic essential functions of the cell related to control of cell growth and differentiation. The products of these genes are detected at different cellular locations and their roles can be divided into four categories namely growth factor, growth factor receptors, nuclear oncogenes and signal transducers (Macdonald *et al.*, 1991). A

mutation of any one of these genes might disrupt the response of the cell to signals which control its growth.

It has been known that DNA viruses such as sv40 virus and RNA viruses such as the retrovirus, Rous sarcoma virus are capable of transforming cells they infect. Most of this information has come from studies of the retroviruses. These RNA viruses encode three genes: *gag*, *pol* and *env* which produce a core protein, a reverse transcriptase and envelope glycoproteins. In those viruses capable of causing malignant transformation, a fourth gene, the oncogene, has been found. Retroviruses can also activate proto-oncogenes more directly by a process known as insertional mutagenesis. In this process, the insertion of a DNA copy of the retrovirus into the cellular genome close to a proto-oncogene is sufficient to cause abnormal activation of that gene.

In model systems it was demonstrated that a single oncogene was insufficient for transformation, collaboration between genes such as *RAS* and *MYC* or *RAS* and *p53* could result in full transformation (Macdonald *et al.*, 1991).

This interaction between the oncogenes is only one of the processes involved in malignant development. Another group of gene, which acts in a growth-regulatory, is also involved in oncogenesis. These genes are tumour suppressor genes.

2.2.2 Tumour suppressor gene

The earliest evidence for tumour suppressor genes pre-dates the discovery of oncogenes by over 20 years. Harris and colleagues (Macdonald *et al.*, 1991) showed that when malignant cells were fused with normal diploid cells, the resulting hybrids were non-tumourigenic as determined by their inability to grow in immunocompromised hosts. This suppression of malignancy was dependent on retention of a specific chromosome. As the hybrids were unstable, there was random loss of chromosome and when a particular chromosome was lost, malignant clones were once again capable of growth. The assumption was that the chromosome that was lost contained the tumour suppressor genes. These genes involved in the control of abnormal growth and whose loss or inactivation is associated with the development of malignancy. Evidence from two types of study supports the existence of tumour suppressor genes in man. These are: (1) the suppression of malignancy in somatic cell hybrids and (2) a consistent loss of chromosomal regions, initially seen in hereditary cancers and subsequently is also shown in sporadic cancers. In the case of retinoblastoma, the probes selected for use were located on 13q (Macdonald *et al.*, 1991). Patients suitable for study were those who had different sized fragments of DNA on each of the two chromosome 13q in their somatic tissue, that is, they were heterozygous. When tumours from the same patients were analysed, only one of the two alleles was present. This loss of heterozygosity can occur by a number of possibly followed by reduplication of the abnormal one, an interstitial deletion of the normal chromosome, or a recombination event resulting in two copies of

the deficient allele. Most of the mechanism shows in result in loss of heterozygosity along the majority of the chromosome.

Some of the tumour suppressor genes functions have been identified. *DCC* and *TGF-β* may be involved in control of cell to cell contact and growth inhibitory factors (Macdonald *et al.*, 1991). Both *RB*1 and *p53* are known to bind DNA and have been shown to act at specific stages of the cell cycle and act as negative regulators of growth by controlling transcription of cell-cycle dependent genes. Mutation of these genes leads to uncontrolled proliferation with aberrant differentiation and eventual tumour formation (Macdonald *et al.*, 1991).

The *p53* gene is located on the short arm of chromosome 17. It is made up of 11 exons covering 16-20 kb of DNA and encodes a 2.2-2.5 kb mRNA producing a 53 kd nuclear protein. This protein is found in most cells of the body and has a short half-life. It has properties of both a tumour suppressor gene and an oncogene. It is found mutated or deleted in a wide range and number of tumours and play an important role in tumourigenesis.

Somatic mutations in the *p53* proto-oncogene are found in approximately 50% of all phosphorylated nuclear protein with DNA-binding properties. It acts as a transcription factor and interacts with a number of other genes. For example, it has been shown recently to activate a gene called *WAF*1, whose protein product halts the cell cycle

in the G1 phase, before DNA replication occurs. This provides the cell with time in which to repair damaged DNA. If *p53* is mutated, cells may replicate damaged DNA. In addition, *p53* is involved in the programmed death of abnormal of damaged cells. *p53* is medically important in at least two ways. First, the presence of *p53* mutations in tumours, particularly those of the breast and colon, signals a more aggressive cancer with relatively poorer survival prospects. Second, *p53* could ultimately prove important in tumour cells results in a significant decrease in tumourigenesis. This indicates that the insertion of normal *p53* into cancer patients' tumours using gene therapy approaches could become an effective cancer treatment (Jorde *et al.*, 1995).

BRCA1 is a gene predisposing to breast and ovarian cancer, was mapped to chromosome 17q21 by linkage analysis. Loss of heterozygosity in breast and ovarian tumours from *BRCA1*-linked patients always involved loss of wild-type alleles from chromosome 17q21, suggesting that *BRCA1* acts as a tumour suppressor gene. It may also act as a tumour suppressor in the far more common cases of sporadic, rather than inherited. Risks of breast cancer to women inheriting *BRCA1* are extremely high, exceeding 50% before age 50 years and reaching 80% by age 65 years (Friedman *et al.*, 1994).

P16 is a candidate for a tumour suppressor gene, which inhibits the cyclin-dependent kinase (cdk) 4 and 6 (Parker *et al.*, 1997). This putative gene is located on chromosome 9p21 and is suggested to be involved in a wide variety of tumour cell lines

and in some primary tumours. It regulates the progression of cells through the G1 phase of the cell cycle (Walker *et al.*, 1994). The cdks phosphorylate the retinoblastoma (Rb) protein, inducing a conformational change and resulting in release of the transcription factor E2F from the Rb protein. Thus, inactivation of either *P16* or *Rb* allows the cell to enter S phase after only a pause at the G1 checkpoint, perpetuating genetic mutations. The homozygous deletions of a region containing the *p16* gene have been detected in many types of human cancer cells, about one-third of which were bladder cancer cell lines. Structural alterations of the *p16* gene have also been shown in lymphoblastoid cell lines derived from dysplastic nervous syndrome, esophageal cancer, pancreatic cancer and primary bladder cancer. (Moyamoto *et al.*, 1995).

2.2.3 Alterations of oncogene in cervical cancer

Chromosome 1 involvement in human cervical carcinoma is of special importance because five oncogenes have been mapped to this chromosome. The oncogenes B-*Lym*-1 and L-*myc* have been localised to band 1p32 (Morton *et al.*,1984). N-*ras* has been assigned to band 1p11-p12, c-*src* to 1p34-p36 (Sreekantaiah *et al.*, 1988) and c-*ski* to 1q22-q24 (Chaganti *et al.*, 1986; Sreekantaiah *et al.*, 1988). Nine fragile sites also have been located on chromosome 1 (1p36, 1p32, 1p31.2, 1p22, 1p21.2, 1q21.3, 1q25.1, 1q42, and 1q44.1) (Berger *et al.*, 1985; Sreekantaiah *et al.*, 1988). Also, genes for nucleic acid synthesis are located on 1q (Helsinki *et al.*, 1985; Sreekantaiah *et al.*, 1988). All of these oncogene sites and fragile at bands or regions usually involved in the

aberrations. However, the role of these oncogenes in the transformation and progression of cervical carcinoma remains to be determined (Wang *et al.*, 1991).

Oncogenes encode proteins that ordinary participate in growth stimulatory pathways in normal cells. Several classes of oncogene products are involved sequentially in transmitting growth stimulatory signals from the periphery of the cell in toward the nucleus. Most studies in cervical cancer have focused on the epidermal growth factor (EGF) receptor and *HER-2/neu.* Epidermal growth factor and its receptor are among the first growth factor/receptor tyrosine kinases to be discovered and characterised at a molecular level. Evidence exists to suggest that overexpression of the EGF receptor may play an important role in the development of some squamous cancers. Several groups have demonstrated that EGF receptor is expressed in normal, dysplastic, and malignant cervical epithelium. It has been suggested that some cervical cancers may overexpress EGF receptor protein (Berchuck *et al.,* 1996).

The *ras* family of G proteins are thought to play a critical role in regulation of cellular proliferation. H-*ras* mutation is a genetic event involved in the pathogenesis of HPV-associated cervical cancers (Berchuck *et al.,* 1996). Structural alterations of the *ras* gene reported to occur in cervical cancer include allelic deletions and point mutations (Vicki *et al.*, 1994). Several reports have shown that cervical dysplasias and cancers have increased *ras* over-expression compared to normal squamous epithelium (Berchuck *et al.,* 1996).

In addition to being a common alteration in early-stage invasive cervical cancers, c-*myc* overexpression may be a relatively early event in cervical carcinogenesis (Devictor *et al.*, 1992). Overexpression of the c-*myc* oncogene, which codes for a DNA-binding protein and is believed to regulate cellular proliferation, has been associated with poor prognosis in patients with cervical cancer (Riou *et al.*, 1992).

Oncogenic viruses are characterised by their ability to integrate into the host genome. It differs from the low risk papillomaviruses in that the viral genomes remain episomal. The high-risk viruses have early gene products known as E6 and E7, which can immortalise human keratinocytes and are responsible for initiating the development of cervical cancer.

The c-*fos* proto-oncogene encodes a nuclear phosphoprotein and is a regulator of transcription, forming a complex with the c-*jun* proto-oncogene product. Overexpression of c-*fos* is common in invasive cervical cancer and correlated with the ability of the tumour to become invasive. It may play an important role in the process of malignant transformation (Cheung *et al.*, 1997).

2.2.4 Alterations of tumour suppressor genes in cervical cancer

Cytogenetic or molecular genetic evidence of chromosome deletion or loss gives important clues to the existence and localisation of tumour suppressor genes. By

demonstrating the common deletion regions in specific tumours, loss of heterozygosity (LOH) studies played a pivotal role in the cloning of tumour suppressor genes associated with tumours (Berchuck *et al., 1996*). Tumour suppressor genes may be more important than oncogenes in cervical carcinogenesis. Human papillomavirus mediates its carcinogenic activity by deactivating *p53* and the retinoblastoma protein, two important antioncogenes (Shingleton *et al.*, 1995). Several investigators have looked for p53 mutation in HPV-negative and HPV-containing cervical cancer cell lines (Ivor *et al.*, 1996; Crook *et al.*, 1991; Scheffner *et al.*, 1991 and Benjamin *et al.*, 1996) and in tissue samples from in situ and invasive cancers. There is a growing body of evidence that inactivation of the wild-type *p53* protein or mutation of the *p53* gene is often associated with the development of cervical cancer (Ivor *et al.*, 1996; Crook *et al,* 1992; Paquette *et al.*, 1993; and Busby-Earle *et al.*, 1994).

2.3 Alterations of chromosome 1 in cervical cancer

2.3.1 Cytogenetic study

In human, there are 46 chromosomes which are arranged in order of decreasing size as 23 matching or homologous pairs. They are divided into the autosomes and the sex chromosomes, which are two X chromosomes in a normal female. One of each pair of the autosomes and one X is of maternal origin and the other 23 are of paternal origin. Each chromosome has a narrow waist called the centromere, which is the site of attachment of the spindle fibres by which the two chromatids are drawn to opposite poles of the spindle during cell division. The centromere divides the chromosome into short

(p) and long (q) arms. Most arms are divided into two or more regions by prominent bands, and each region is further subdivided according to the number of visible bands. The tip of each arm is the telomere. Chromosomes 1 are metacentric. Of all the chromosome, chromosome 1 is the most frequently involved in human cancer and leukaemia. Its relationship to the malignant process has been stressed repeatedly. Atkin and Baker described 26 cases of cervical carcinoma with numerical and structural alterations of chromosome 1. This includes short arm deletions, long arm isochromosomes and translocations of unidentified chromosomal material onto one of the arms (Atkin *et al.*, 1978). Cytogenetic analyses have revealed chromosome 1 as a predominant target for alterations in cervical carcinomas (Sreekantaiah *et al.*, 1988; Schwab *et al.*, 1996), particularly band p35-36 (Zimonjic *et al.*, 1995; Schwab *et al.*, 1996). Specific regions of chromosome 1, on both the short arm and long arm, were present consistently in the marker chromosomes formed. Region 1p11-1p13 was retained in 84% of the rearrangements and region 1q21-1q32 in 94% of the changes. (Sreekantaiah *et al.*, 1988). Deletions following breakage at various sites in the short arm of chromosome 1 are frequent in malignancies and are quite often found in cells that are trisomic for the long arm. Band 1q32 and 1p36 was the most frequently involved breakpoint in deletions, inversions, translocations, and duplications (Sreekantaiah *et al.*, 1988). It is suggested that fragility of chromosomes generated as a result of early events in carcinogenesis may promote rearrangements, including those of chromosome 1, and give rise to progressively more malignant clones (Brito-Babapulle *et al.*, 1981).

2.3.2 Molecular genetic study

Chromosome 1 is the largest of the human chromosomes (Stephen *et al.*, 1990; Schwab *et al.*, 1996). Its physical length of the short arm and long arm are 128 and 135Mb respectively (Morton *et al.*, 1991; Schwab *et al.*, 1996). A considerable number of tumour suppressor genes have been isolated and characterised. However, more tumour suppressor genes remain to be identified and isolated. The most typical approach in isolating a tumour suppressor gene is so-called "positional cloning". This is based on Takayama *et al.*, 1992; Caron *et al.*, 1993; Schwab *et al.*, 1996). The frequency of LOH is 26% at 1q (Mitra *et al.*, 1994). LOH at D1S76 and D1S80 loci on chromosome 1p36 occurs in 26 of 44 (59%) invasive cervical carcinoma (Wong *et al.*, 1993). Chromosomal changes may be the first clue through which investigators discover precise molecular changes resulting in malignant transformation.

2.4 Loss of heterozygosity (LOH)

Loss of heterozygosity is diagnosed when an allelic band present in the constitutive cell lane is absent in the tumour lane (Peter *et al.*, 1992). Detection of LOH allows a precise mapping of minimal deleted regions in tumours. It is an essential step toward the cloning of a tumour-suppressor gene and has been linked with tumour aggressiveness in various tumour types. The genotyping of tumours can be made an important parameter in the pretherapeutic evaluation of cancer patients, provided that routine analyses of these alterations rely on simple and efficient method suitable for the analysis of the small biopsies presently used (Peter *et al.*, 1992). Wong *et al.* (1993)

previously observed LOH at the DIS76 and DIS80 loci on chromosome 1p36 in 26 of 44 (59%) invasive cervical carcinoma. Further construction of a detailed mapping of genetic deletion in cervical cancer will be invaluable for identification of the tumour suppressor gene.

LOH of specific chromosomal segments occurs in many neoplasias and generally indicates the sites of tumourigenicity suppressor-like genes. Loss of the wild-type function of these genes appears to be a critical step on the origin or progression of the malignant phenotype (Michael *et al.*, 1996). Deletion or somatic LOH in the tumour tissue has been demonstrated at loci on distal chromosome 1p in some melanomas, medullary thyroid carcinomas, phaeochromocytomas (Brodeur *et al.*, 1990), neuroblastomas and breast cancer.

Usually, there are two methods for analysis of LOH. They are polymerase chain reaction (PCR) and Southern blot hybridizayion. For PCR, all primers can be labelled with radioactivity before amplification reaction and primer sets specific for each locus are used to amplify the repeats and short flanking sequences in template DNA, followed by separation in denaturing 8% polyacrylamide-urea-formamide gels followed by autoradiography at -70°C. For Southern blotting, DNA is extracted from blood and tissue samples using standard phenol and chloroform extraction, followed by ethanol precipitation, restriction enzyme-digested DNA, size separate in an agarose gel, is blotted onto a nylon membrane and hybridised to available number of tandem repeat probes.

Probes are labelled with radioactive using the random primer technique. Following high-stringency washing at 65°C in 0.1 x standard saline citrate (SSC) plus 0.1% SDS, membranes are analysed by autoradiography. Thus Southern blotting approach requires relatively large amounts of purified DNA, usually several micrograms (up to 1 ml of fresh blood is needed for every procedure) and several days to set up an experiment. Conversely, PCR is a more sensitive method that can amplify even a single copy of a target sequence on a given sample (Saiki *et al.*, 1985). LOH studies are very useful in identifying specific chromosomal regions of loss in small archival samples (Rader *et al.*, 1996). It is a rapid method allowing many samples to be handled at one time. In view of these advantages, we implemented PCR method in our study.

Chapter Three

Materials and Methods

3.1 Materials

3.1.1 Patients

One hundred patients with cervical cancer recruited from the Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Prince of Wales Hospital, were included in this study.

3.1.2 Specimens

3.1.2.1 Blood samples

20 ml of peripheral blood samples from each patient were obtained at the time or within a few days of operation, and collected into a tube containing 2.5 ml of sodium citrate (3.8%) for DNA extraction.

3.1.2.2 Tumour tissue specimens

Tumour tissue from 100 cases of cervical cancer were collected. A representative part of the tumour tissue was initially dissected. Most of the tissue was snap-frozen in liquid nitrogen and stored at -70°C pending DNA extraction. The remainder was embedded in OCT compound for frozen section. The sections were stained with hemotoxylin and eosin, and were used to corroborate the histological diagnosis and determine the proportion of tumour to stromal tissue in the specimen

collected. The proportion of malignant cells in all of tumour tissues used in this study was more than 50%. The histological type and grades of the tumours were classified according to the criteria of the World Health Organisation (Shingleton *et al.*, 1995). The stage of cancer was established according to International Federation of Gynaecology and Obstetrics (FIGO) criteria (Creaseman *et al.*, 1988).

3.1.3 Chemicals and reagents

3.1.3.1 Chemicals

1) Acrylamide (GibcoBRL, Grand Island. N,Y, USA.)

2) Agarose (Sigma, St. Louis, USA.)

3) Ammonium Persulfate (Sigma, St. Louis, USA.)

4) Bisacrylamide (Sigma, St. Louis, USA.)

5) Boric acid (Ultra pure) (GibcoBRL, Grand Island. N,Y, USA.)

6) Bromphenol blue (Sigma, St. Louis, USA.)

7) Cell Lysis Buffer (Cruachem, Glasgow, UK.)

8) Ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, USA.)

9) Formamide (Amresco, Ohio, USA.)

10) 8-hydroxyquinoline (E. Merck, Darmstadt. F. R, Germany.)

11) 2-mercaptoethanol (E. Merck, Darmstadt. F. R, Germany.)

12) Phenol (Ultra pure) (GibecoBRL, Grand Island. N, Y, USA.)

13) Sodium dodecyle sulfate (SDS) (GibecoBRL, Grand Island. N, Y, USA.)

14) Phenol: Chloroform:Isoamy Alcohol (25:24:1) (GibcoBRL, Grand Island. N, Y, USA.)

15) Sodium chloride (Amresco, Ohio, USA.)

16) Taq DNA polymerase (5U/μl) with magnesium chloride and 10 x Taq DNA polymerase buffer (GibcoBRL, Grand Island. N, Y, USA.)

17) T4 polynucleotide kinase with 5 x forward reaction buffer and 5 x exchange reaction buffer (GibcoBRL, Grand Island. N, Y, USA.)

18) Taqstart antibody (Boehringer Mannheim, San Louis, Obosco, USA.)

19) Tris (hydrexymethyl) aminomethane (Sigma, St. Louis, USA.)

20) TEMED (N,N,N',N',-Tetramethlethulenediamine) (GibcoBRL, Grand Island. N Y, USA.)

21) ϕX-174 -RF DNA (GibcoBRL, Grand Island. NY, USA.)

22) dNTP set (2'-deoxynucleoside 5'-triphosphate) (Pharmacia Biotech, Uppsala, Sweden.)

23) [γ32p]ATP (10 mCi/ml) (Amersham, Buckinghamshire, UK.)

24) Urea (Enzyme Grade) (GibcoBRL, Grand Island. N, Y, USA.)

25) Xylene cyanol (Sigma, St. Louis, USA.)

3.1.3.2 Reagents

1) Buffered Phenol

phenol	500 g
2 M Tris (hydrexymethyl) aminomethane	200 ml
m-cresol	25 ml
2-mercaptoethanol	1 ml
8-hydroxyquinoline	0.5 g

2) Cell lysis buffer

Sucrose	0.32 M
Tris-HCL, PH7.5	10 mM
MgCL2	5 mM
Triton X-100	1%

3) DNA extraction buffer

Proteinase K	200 μg/ml
NaCl	10 mM
Tris (hydroxymethyl) aminomethane	10 mM
Ethylenediaminetetraacetic acid (EDTA)	25 mM
Sodium dodecyle Sulfate (SDS)	0.1%

4) 10 x TBE electrophoresis buffer

Tris base	60.5 g
Boric acid	30.85 g
0.5 M EDTA, PH8.0	3.72 g
Add sterile distilled water to 1L	

5) DNA molecular weight marker (10.5 μl)

5 x exchange reaction buffer	2 μl
ϕX174 RF DNA/Hae III Fragments (50 ng/μl)	2 μl
Sterile distilled water	5.1 μl
T4 Polynucleotide Kinase (1U/μl)	0.4 μl
[γ32p]ATP (10 mCi/ml)	1 μl

6) 32% formamide-8% polyacrylamide gel (60 ml)

Acrylamide solution 40% (Acrylamide:Bis-acrylamide=19:1)	14ml
Formamide	19.2 ml
Urea	20.16g
TEMED	36μl
5 x TBE buffer	6 ml
Ammonium persulfate 10%	660 μl
Add sterile distilled water to 60ml	

7) 2 x loading solution (10ml)

EDTA	10 mM
Bromphenol blue	0.01 g
Xylene cyanol	0.01 g
Add formamide to 10ml	

3.1.3.3 Markers

Twenty-nine microsatellite markers and two variable number of tandem repeats markers located on chromosome 1, were used in this study. The sequences and mapping of primer pairs are shown in Table 3.1-2 and Fig 3.1 respectively. These markers were purchased from or synthesised by Research Genetics, Inc. Memorial Parkway, USA.

Table 3.1. Oligonucleotide primers used in this study

Name of primer	Locus symbol	Size range of product (bp)	Sequences
AFMa210xg9	D1S2663	183-205	5'-ATGACTGTTCTCTGTTTTAGGAACC-3'
			5'-CAGTTGTTGGGAAGGCG-3'
AFMa127zc9	D1S507	183-203	5'-AGGGGATCTTGGCACTTGG-3'
			5'-CTCTAGGGTTTCTGGAAAATGCTG-3'
AFM200wh8	D1S2722	195-223	5'-CAAATAATGCTACCATTGC-3'
			5'-TTCTGGTCATTTACCCTG-3'
AFMA082wb5	D1S2890	175-193	5'-TGCTGGACATTTAGATGTAATGAA-3'
			5'-GGTAGGACTGTTGTGAAGATGG-3'
AFM303vc5	D1S2829	199-225	5'-AGTGGTTTATATGACTTACTGTGGG-3'
			5'-GCACNCCAGCCTAGGTA-3'
AFM113xf6	D1S206	206-218	5'-GGAAGTAATCTCTGCCCAAT-3'
			5'-CCAAAGTTTCTTCTGGTATTTGT-3'
AFMa133ye5	D1S2635	142-159	5'- TAGCAGATCCCCCGTC-3'
			5'-TGAATCCTACCCCTAAGTAGAAT -3'
AFMa057ze5	D1S2878	169-195	5'-TATTGGCTGAGGATGAGGTT-3'
			5'-ACCAGANCCACAATCTCTAAAC-3'
AFMb359xf5	D1S2799	191-209	5'-AGCAAGACCCTGTCTCAAAA-3'
			5'-TGGATAGCTTTCCACCACT-3'
AFMa285zd5	D1S2691	249-267	5'-AGCTGGATATGGCTCAA-3'
			5'-CGCTGGTAGCCTTTAATG-3'
AFM205xg1	D1S238	272-302	5'-TCATGTCTAGATCCTGTGCC-3'
			5'-TGGAGGCAGTTTAGATTGTG-3'
AFMb309xe1	D1S2757	253-271	5'-TTTTTTAATGACTGACCAGTG-3'
			5'-TGCCTTCTGCTATGTTTG-3'
AFMa190xd5	D1S2655	224-260	5'-AGGGTCCCCAAAGAGCCTTC-3'
			5'-ATGGCAGCACATCCTGCTTC-3'
AFMa290xd1	D1S2692	187-217	5'-GCTAACAAAAACCCACATCT-3'
			5'-CTATAAGCAAATGGCACAAG-3'
AFMa303wh5	D1S2703	196-228	5'-AGCTCTCCAAGTCGGGATAG-3'
			5'-CATTTACCCCAGTGTTGCAG-3'
AFM321xe5	D1S2833	92-108	5'-ATGAATACCACATAAAATGTGTATG-3'
			5'-CTGAGCCCCAGATTGAC-3'
AFMa162zc9	D1S2647	177-193	5'-AACAACGGCAAGACTCCATC-3'
			5'-TACCTCCGCCCTGAAATG-3'
AFMa048yh1	D1S2864	141-167	5'-AGCTCTAATGCCCGAAACC-3'
			5'-TCTTTCATTCAGTCGCTCCC-3'
AFMa190zc5	D1S2656	182-210	5'-TGCTTGGAAGTCATGGAAC-3'
			5'-CTAGGCAAAAATCATCTGGGTGC-3'
AFMa140yh1	D1S2638	224-244	5'-CTTGGATTGGTGGGTACTA-3'
			5'-AGGTTTCAGGGTGGCT-3'
AFM344we9	D1S2845	193-223	5'-CCAAAGGGTGCTTCTC-3'
			5'-GTGGCATTCCAACCTC-3'
AFMa060yh9	D1S2882	224-237	5'-AATGAAAATTGTAGTACCTGTTCG-3'
			5'-CTTGCTAAGGATGATAGCCTC-3'
AFM350tg9	D1S2849	174-184	5'-AGCTGAGATCGTGCCA-3'
			5'-TCCCTAACCCTCCAGACT-3'
AFM207yh6	D1S2346	89-115	5'-TATCTTGCCCYGCACC-3'
			5'-AAGTGGGTCTCCCCAG-3'
AFMb359xb9	D1S2785	164-178	5'-CGTGAATATCCTCAGGGAAT-3'
			5'-ATTGTGGCACCGTACTCC-3'
AFM123yc7	D1S461	234-246	5'-TTACTGCACTCCTGTCTGGG-3'
			5'-TGGGCTATCAGAAGCCTCTT-3'
AFM164yg1	D1S222	258-276	5'-GCCTTCTGGCTCTGAAACTC-3'
			5'-CTGAAGAACCCGCTATGAAG-3'
AFM095ta5	D1S202	77-91	5'-CAACACCCAAACAGATGACC-3'
			5'-AGTCTTTCATGGCCACTGTG-3'
AFM031xd12	D1S412	185-207	5'-TAGGACTTTTCAACTTCCACAG-3'
			5'-ATAGGCACAGAATCAATGAATG-3'
76.10	D1S76	350-1700	5'-GGGTCTCAGCGGATGAAGGTTTTTG-3'
76.11			5'-GAACGTGTCACTGATGTAGCGGTTG-3'
80.3	D1S80	387-1000	5'-TGCGTGTGAATGACCCAGGAGCGTAT-3'
80.4			5'-TCTGGCTTGTTTATTTTGTCTTGTTGGAG-3'

Table 3.2 Oligonucleotide primers used in this study

Name of primer	Locus symbol	Map	Annelling temperature (°c)
AFM344we9	D1S2845	1p36.2	55
AFMa210xg9	D1S2663	1p36.3	55
AFMa127zc9	D1S507	1p36.3	55
AFMa162zc9	D1S2647	1p36.1	59
AFMa048yh1	D1S2864	1p34.3	59
AFMa190zc5	D1S2656	1p34.3	59
AFM200wh8	D1S2722	1p36.1	57
AFMa082wb5	D1S2890	1p33	57
AFMa140yh1	D1S2638	1p34.1	59
AFM303vc5	D1S2829	1p31	57
AFMa060yh9	D1S2882	1p33	59
AFM350tg9	D1S2849	1p31	59
AFM113xf6	D1S206	1p21	55
AFM207yh6	D1S2346	1p13	59
AFMa133ye5	D1S2635	1q12	57
AFMa057ze5	D1S2878	1q21	55
AFMb359xf5	D1S2799	1q21	57
AFMa285zd5	D1S2691	1q24	55
AFM095ta5	D1S202	1q31	55
AFM205xg1	D1S238	1q25	55
AFMb309xe1	D1S2757	1q25	57
AFMa190xd5	D1S2655	1q25	57
AFM031xd12	D1S412	1q25	55
AFMa290xd1	D1S2692	1q32	55
AFMa303wh5	D1S2703	1q31	59
AFM321xe5	D1S2833	1q41	59
AFMb349xb	D1S2785	1q42	59
AFM123yc7	D1S461	1q42	55
AFM164YG1	D1S222	1q42	55
76.10 76.11	D1S76	1p36-1p35	66
80.3 80.4	D1S80	1p36-1p35	57

Fig 3.1 The location of markers on chromosome 1

3.1.3.4 Major equipment

1) Spectrophotometer, DU 650 (Beckman, USA.)

2) Programmable thermal controller, PTC-100TM (M. J. Massachusetts, USA.)

3) Sequencing gel electrophoresis apparatus, Model S2 (GibcoBRL, Grand Island. N, Y, USA.)

4) Slab gel dryer, SE 1160 Drygel, SR. (Hoefer, San Francisco, USA.)

5) Imaging Densitometer (Model GS-670) (Bio-Rad Laboratories, CA, USA.)

3.2 Methodology

3.2.1 DNA extraction

Beginning with blood

- Added Lysis Buffer to each 50 ml tube (1:10 dilution), then, inverted gently until lysis was completed (about 3 min)
- Centrifuged at 3,200 rpm for 15 min at 4 °C.
- Decanted supernatant, then, resuspended pellet in 4.5 ml TE buffer.
- Added 0.5 ml 10% SDS and 100 µl Proteinase K (20 mg/ml). Set in 65 °C water bath until clear.

Beginning with tumour tissue

- Removed the tissue from -70 °C, then, minced the piece of tumour in a tube with TE buffer.

- Volumes for the digests could vary but a good starting point was 170 µl TE buffer, 20 µl 10% SDS, 10 µl Proteinase K (20 mg/ml).

-Incubated the sample at 65 °C until it has become clear and the small pieces digested.

Extraction of nucleic acid

- Added 5-10 ml phenol/chloroform/isomyalcohol (24/25/1), inverted gently to emulsify, and centrifuged at 2,800 rpm for 2 min at room temperature.

- Aspirated top aqueous layer with pipette and transferred to 2nd tube containing 5-10 ml phenol/chloroform/isomylalcohol (25/24/1), inverted gently to emulsify, and then centrifuged as in 1).

- Transferred aqueous layer to a fresh tube, added 15 ml of chloroform/isoamylalcohol (24/1), and mixed. Then centrifuged at 3,200 rpm for 1 min.

- Transferred the aqueous (top) layer to a new tube and added ½ v of 7.5 M ammonium acetate and 2 (original) v of 100% ethanol. Recovered DNA by centrifugation at 5,000 rpm for 2 min.

- Rinsed the pellet with 70% ethanol. Decanted ethanol and vacuum dried the pellet.

- Resuspended the pellet with sterile distilled water until dissolve, and determined its concentration.

3.2.2 DNA amplification

3.2.2.1 Validation of PCR primer and optimization of PCR condition

Initially PCR amplification except for loci D1S76 and D1S80 was carried out in a Perkin-Elmer 9600 thermal cycler with 50 ng normal female blood DNA in a total volume of 25 μl solution containing 0.25 μM of each primer (The different pairs of primers described in Table 1 were included in one reaction), 10 x PCR buffer (200 mM Tris-HCl and 500 mM KCl), 1.5 mM MgCl2, 100 μM of each dNTPs , 1 μl of 50% DMSO, 0.47 unit of *Taq* DNA polymerase. The reaction started an initial denaturation step at 94°C for 3 min followed by 35 cycle at 94°C for 1 min, at 55°C for 1 min and at 72°C for 1 min lastly for a final extension by 10 min at 72°C. The concentration of DMSO and annealing temperature were adjusted until the clear expected band in gel was available.

3.2.2.2 End labelling of the primers by [γ-32p]ATP

- Mixed the followings in a sterile 0.6ml microcentrafuge tube:

5 x forward kinase buffer	9.8 μl
Primer (20 μM)	0.9 μl
Sterile distilled water	32.624 μl
T4 Polynucleotide kinase (1U/μl)	1.176 μl
[γ-32p]ATP (10 mCi/ml)	0.5 μl
Total volume	49 μl

- Incubated the reaction mixture at 37°C for 30 min.

- Stopped the reaction at 65°C for 10 min

- Transferred the reaction mixture into the master mix .

3.2.2.3 PCR for LOH detection

- Preparation of PCR 49x master mix except for loci D1S76 and D1S80:

Sterile distilled water	909.6 µl
Taq DNA polymerase (5 U/µl)	6.125 µl
Taqstart antibody (1.1 µg/µl)	3.0625 µl
Forward primer (10 µM)	12.25 µl
Reverse primer (10 µM)	12.25 µl
dNTP (10 mM)	24.5 µl
10 x Taq DNA polymerase buffer	122.5 µl
Magnesium chloride	36.75 µl
Total	1127 µl

- Mixed the mix well. Before aliquot 23 µl master mix into each numbered 0.6 ml tube, added 2 µl of sample DNA (50 ηg/µl) to the appropriate tube.

- Mixed the reaction mixture and centrifuged briefly.

- Placed the tubes in a PC-100 programmable thermal cycler and ran in as the following conditions:

Step 1 94°C 3 min

Step 2 94°C 1 min

Step 3 55~59°C 1 min (Table 2 showed different temperature for different primer)

Step 4 72°C 1 min

The step 2 ~ 4 were repeated for 34 times.

Step 5 72°C 10 min

steps 6 10°C keep on

3.2.2.4 Electrophoresis

- 8% urea-formanide-denaturing-polyacrylamide gel was used.

- Prepared the glass plates and spacers for pouring the gel. Before running the gel, treated one surface of the short glass plate with a gel plate coating, this prevented the gel from sticking tightly to the plate. The two plates were of slightly different size and one of them was notched. Laid the longer plate flat on the bench and rinsed plates with ethanol. Arranged the spacers at each side parallel to the edges. Laid another plate in position, resting on the spacer bars. Binded the entire length of the two sides and the bottom of the plates with gel casting clamp (Life Techologies, USA).

- Poured the gel into the space between the two glass plates. Inserted the comb on the top of the gel. Clamped the comb in place with three clips and allowed the acrylamide to

polymerise for 60 min at room temperature.

- When the gel had polymerised, carefully removed the comb. Attached the gel to the electrophoresis tank, the notched plate should face toward the buffer reservoir. Filled the reservoirs of the electrophoresis tank with 0.5 x TBE (1000ml). Rinsed out the wells with 0.5 x TBE at once with pipette. Reinserted the comb between the plates with teeth down.

- Checked for leakage. Connected the electrodes to a power pack and pre-run for 30 min.

- Mixed 3 μl of the DNA for each sample with the appropriate amount of 2 x loading buffer. Loaded the mixture into the wells .

- Connected the electrodes to a power pack and ran electrophoresis at 45 w for 3 h.

3.2.2.5 Gel dry and radioautography

- Ran the gel until the marker dyes had migrated the desired distance. Turned off the electric power.

- Using the 3M gel casting tape to detach the short glass plates from the gel, and removed the spacers. Cut a piece of 3 mm Whitman paper to approximate gel size and laid it on the gel. Peeled the gel off the plate.

- Warped the gel with Saran Wrap. Cut another piece of 3 mm Whitman paper and placed on gel dryer. Then, putted the gel above it.

- Turned on the vacuum and heated the gel at 80°C for 20 min. Exposed the dried gel to Kodak x-ray film for over night at -70°C and developed.

3.2.2.6 PCR analysis of the D1S80 and D1S76 loci

PCR amplification was carried out in a Perkin-Elmer 9600 thermal cycler with 50 ng normal female blood DNA in a total volume of 25 µl solution containing 0.5 µM of each primer, 10 x PCR buffer (200 mM Tris-HCl and 500 mM KCl), 1.5 mM MgCl2, 100 µM of each dNTPs, 1 µl formamide, 1.0 U of platinum *Taq* DNA polymerase. A total of 30 cycles of PCR were carried out, each cycle consisting of denaturation at 94°C for 0.5 min, annealing for 1 min at 66°C for D1S76 or 57°C for D1S80, and elongation at 72°C for 2.5 min, which was preceded by 5 min at 94°C and followed by a final extension by 5 min at 72°C. The resulting amplified fragments were identified after 1.2% agarose gel electrophoresis and ethidium bromide staining.

3.3 Determination of loss of heterozygosity (LOH)

The signal intensity of the alleles in constitutionally heterozygous samples would been analysed by using an Imaging Densitometer. LOH of tumour samples was determined by comparing the intensities of bands obtained from tumour DNA with that from matched blood DNA. Only the case with 70% or more reduction of intensity in one band from tumour sample compared to that from blood sample, was considered to be positive for LOH. Tumour tissues inevitably contain stromal cells, which might lead to erroneous interpretation of the results. The proportion of malignant

cells in the tumour samples studied was more than 50%. Theoretically, therefore, the samples can be considered to have heterozygous deletions, if the ratio is lower than 0.5. In this study, heterozygous deletions were assigned more strictly to tumours in which the ratio was reduced to more than 70% of the ratio in matched blood DNA. The positive result was confirmed by a second PCR and electrophoretic run.

3.4 Statistical Analyse

Statistical analysis of possible correlation between observed LOH and patient's age, grade and stage at diagnosis, and clinical status were performed using Pearson's chi-square test and Fisher's exact test.

Chapter Four

Results

4.1 LOH analysis in cervical cancer

LOH of tumour samples was determined by comparing the intensities of bands obtained from tumour DNA with that from matched blood DNA. Only the case with 70% or more reduction of intensity in one band from tumour sample compared to that from blood sample was considered to be positive for LOH. We evaluated 31 loci covering the whole chromosome 1 in 100 cervical cancers. The markers mapping within the high rate of heterozygosity were selected to maximise the information of this analysis. The positive result was confirmed by a second PCR and electrophoretic run.

Tables 4.1 shows frequency of LOH at 31 loci on chromosome 1 in 100 cervical carcinomas. Fig 4.1 shows LOH detected at different loci on chromosome 1 in 100 cervical cancers, in details.

The number of tumours informative for each locus ranged from 40 (40 %) to 95 (95%). LOH was detected in 96 of 100 (96%) cases of cervical cancer. LOH at two or more than two loci was detected in 88 of 95 (93%) informative cervical carcinomas, while 8 of 95 cases (8.4%) had LOH detected only at one locus.

Among the 31 markers examined, the highest incidence of LOH was at locus D1S80 (45%). The second most frequent LOH was at D1S2829 (44%).

D1S76 also showed frequent LOH (43%). The rate of LOH at loci D1S2663 and D1S2757 was 35% and 31% respectively. Another 12 loci were involved in LOH in 20-28% of the informative tumours. All other loci had LOH in 20% or less of the informative cases. Therefore, the critical region appeared to centre around the loci D1S80, D1S76, D1S2663, D1S2829 and D1S2757 at 1p36-35, 1p36.3, 1p31 and 1q25 respectively (Fig 4.2).

Sample No.	Markers																															
	D1S2890	D1S2829	D1S2757	D1S2703	D1S2635	D1S2878	D1S2655	D1S2833	D1S2882	D1S2849	D1S2647	D1S2845	D1S461	D1S202	D1S222	D1S412	D1S2799	D1S2663	D1S2346	D1S2722	D1S2785	D1S2638	D1S2864	D1S2656	D1S238	D1S507	D1S206	D1S2691	D1S2692	D1S76	D1S80
C219	o	●		o			o	o		o	o	o		o	o	o	o	o	o				o	o	o	●	o	o	o	●	o
C223			o	o		o	o	o	o	o	o			o					●	o		o	o	o	o	o	o		o	●	o
C224			o	●	o	o	o	o		o	o		o	o	o	o				o	o	o	o		●	o	●	o	o	o	o	●
C225	o			o		o				o	o			o	o	o			o		o		o	o	o	o	o	o	o	●	o
C228	o		o			o	o	o	o		o	o		o		o	o		●	o	o				o	o	o	o	o	o	●
C230	o	●		o		o	o	●	o		o	o		o	o	o	o		●	o			●	●	o	o	o	o	o	●	●
C231	o	o	o	o	o		o	o	o	o	o		o	o		o			●		o			●	o	●	o	●	o	●	o
C232	o	o	●	o		o		●	o		●	o			o	o	●		o		o	o		o	o	o	o		o	o	●
C235	o			o				o				o	o	o		o	o	o	●	●	o	o	●	●	o	o	o	o	o	●	o
C236	o	o	●	o	o	o		o	o		●	o	o	o	o		o		●	o	o	●			o	●	●	o	o	●	o
C241	o	o		o		o	o	o		o	●			o	o	o	o		●	o	o	o	o	o	●	o	o	o	o	o	●
C242				o	o	o		o		o	●	o	●	o	o	o	o	o			o	o	o	o	o	o	●		o	o	o
C244	o	o	●	o		o		o	o		●			o	o	o	●	o	o		o		●	o	o	o	●		o	o	●
C246		●			o	o		o		o	o	o	o	o	o	o	o		●	o	o			o	o	o	o		o	●	o
C247	o	o		o		o	●	o	o	o	o	o	o	o	o		o	o	o	o	o	o	o	o	●	o	o	o	o	o	●
C250	●	o				●	●	o	o	●	o	o	●	o		o			●			●	o	o	o	●	o			●	●
C253					●		o	o	●		●	●		o		o		●	●			o	●		o	o	●	o	o	●	o
C254	o	o	o	o	o	o	o	o	o		●	o		o		o			o	o		o	o	●	o	o			o	●	o
C255	o	o		o	o	o		o	o	o	o	o	o	o	o	o		o	o			o	o	o	o	o		o	o	o	o
C256	o	o		o		o		o		o		o		●	o	o		o	o		o		o	●	o	o	o		o	●	
C257	o	●		o		o	o	o					●	o	o	o	o	o	●			o		●	o		o	o	o	o	o
C259			o	o			o	o				o	o	o		o	o	●	o		o	●		●	o	o	o		o	●	o
C261	o						o	o	o	o	o			o	o		o		o	o		o	o	o	o	o	o	o	o	●	o
C265	o	●					●	o				●			o	o		●	o	o	●			o	●	●		o		o	●
C269	o			o	o				o	o		o	o	o		o			●		●				o		o	o	o	o	o
C270			o	o	o	o		o	o	o	o	o	o	o	o	o	●		o	o	o			o	o	o	o	o	o	●	o
C271	o	●		●	●	●	o	●	o	o	●			o	o				o		●	o		o	o		o	o	●	o	
C273	o	●	o	o	o	o		o	●	o				o	o	o	o	o	o		o				●	o	o		o	●		
C275	●	o		o	o	o	o	o	●	o	o	o		o	o	o	o	o	o		●		●	o	●	o	o	o	o	o	o
C276	o		o		o		o		o	o		o		●		o			o		o			o	o	●	o		o	o	
C277	o					●		o	o	o			o	o			o		●			o	o		o	o	o			o	
C278	o			o	o			o		o	o	o	o	o	o			o	o		o		o		o	●	o		o	o	●
C282	o	●		o	o	o	o		o			o	o	●		o	o	o	o	o	o					o	o		o	o	
C283	●		●	●	o	●	●	o	o	o		o	●	o			o		●			o		o	o	o	o	o	o	o	

Figure 4.1 LOH pattern on chromosome 1 in cervical cancer

•: Informative case with LOH o: Informative case without LOH Blank: Uninformative case.

continue

Sample No.	Markers																															
	D1S2890	D1S2829	D1S2757	D1S2703	D1S2635	D1S2878	D1S2655	D1S2833	D1S2882	D1S2849	D1S2647	D1S2845	D1S461	D1S202	D1S222	D1S412	D1S2799	D1S2663	D1S2346	D1S2722	D1S2785	D1S2638	D1S2864	D1S2656	D1S238	D1S507	D1S206	D1S2691	D1S2692	D1S76	D1S80
C284			o	o		●	●	o	o	o	o		o	o	o		o	o	o			o			o	o	o	o		o	o
C285		o	o		o				o			o		o	o	o		o	o		o				o	●	o		o	o	o
C289	o			o			●	o	o			o		●	o	o	o		o		o	o	●		o	o	o			●	
C290		●	o		o		o	o	o	o	o	o		o		o	●		o	o	o				o	o	o			●	o
C291	o		●	●		●		o		o	o	o		o			o	o	o	o			o	o	o	o	o		●		
C292	●			o	o		o	o			o	o		o	o		o	●	o	o	o		o	o	o	o	o	o	o	o	
C298	o	o		●		o		●	o		●		o	o	o	o		●	o			o			o	o	●	o		o	●
C299	o		o	o			o	o	o		o	o	o	o	o	o			o	●	o	●	●	o	o	o	o		●		o
C300	o			o			o		o	o	o		o	o	o	o			●			o	o	o	o	o	o	●		o	o
C301	o			o		o	o		o	o		o		o			●	●	o	●	o	o		o	o	o	o			●	●
C302		●	o		o		o		o		o	o		o	o	o	●		o		●	o		o	o		●		o	o	o
C304								o	o	o	●	o	o	o	o		o		o		o	o			o		o			o	●
C305	●	o	●	●		●	●	o		●			●	o	●	●		●	o		●			o	o		●	o	●	●	●
C307	●	o	o			o	o	o		o	●		o	o	o	o		●	o	o	o	●		o	o		●		o	●	o
C308	o		●	o		o		o	o	●		o	o	o	o		●		o		o	o	o	●	o		●		o	o	
C312	o	o	●	●		o		o	o	o	o		●	o	o	o	o	●	o	o	o	o	o		o	o	o		o	o	●
C316		o	●	o		●	●			o	o	o	●	●	●	o		●	o		o			●	o	o	●	o	o	●	o
C317	o		●	o		o		o			o	o		o	●	o			o		o	o		o	●	●			o	o	●
C318			●	●	●			●	o			●	o	●	●	o		o	o				●		o		●	o	o		
C321	o	o	o				●			o		o	o	o	●	o	●	●	●		o				o	●	●				●
C325				o		o		o	o	●		o		o		o			o			o	●		o	o	o		o	o	●
C327	o			o	o			o	●			o	o	o	o	o				o		o			●	o	o		o	o	o
C328		o		o			o	●		o	o	o	o	●	o	●	●	●	●						o	o	●	●	●		o
C334	●			o	o		o	o	o	o		o		o		o		o	o		o	o	●	o	o	●	o				o
C335	●	o	o	o		●	●	o				o		●		●		o	o		o	o			●	o	o		o	●	●
C336	●	●	o	o		o	o	o		●	●	●	o	o		o			o	o	o				o	o	o		o		●
C338	o	●	o				o	●	o	o	o	o	o	o		o			o	o					o	o	o		o		●
C340	o		o	o		●	●	●	o	o		o	o	o	●	●	●	o	o	o				o	●	o		o			
C343		●	●	●		●	o	o	o		o	●	●	●	o	o			●		●	●	o		o		o	o			●
C345			o			o	o				o	●	o	o	o	o			●		o				o		●	o	o	●	o
C346		o			o	o			o		o		o	o		o	o		o	o		o	o		o			o		o	o
C348		o			o	o		o	o		o	o		o		o	o	o	o	o					o			o	●	o	o
C349	●	o	o	o				●	o		o	o	o	o		o	●	o	o		o	o		●	●		●	o	o	o	
C350	o		o	o	●	o	o	●	o	o		o	o	o		o	●		●	●			o	●	o			o	o	●	o

Figure 4.1 LOH pattern on chromosome 1 in cervical cancer

•: Informative case with LOH o: Informative case without LOH Blank: Uninformative case.

Continue

Sample No.	Markers																													
	D1S2890	D1S2829	D1S2757	D1S2703	D1S2635	D1S2878	D1S2655	D1S2833	D1S2882	D1S2849	D1S2647	D1S2845	D1S461	D1S202	D1S222	D1S412	D1S2799	D1S2663	D1S2346	D1S2722	D1S2785	D1S2638	D1S2864	D1S2656	D1S238	D1S507	D1S206	D1S2691	D1S2692	D1S76	D1S80
C354				o	o	o	o		o		o	o	o	o		o	o		o		o	o	o			●	o	o			o
C356	o	●	o		o	●	o		o		●	●	o	o		o			o	●	●		o			o	o	o			●
C358		●			●		●			o	o	o	o	o	●	●			●			●			o	●	o	o			●
C361		●		●				o					o	o	●	o		●	o		●			o	o	o	o	●		o	
C365	o		o	o		o		o		●		o		o		o	o		o			o	o	o	o	●		o		●	o
C366	o		o	o	o	o		o		o	o	o	o			o	o	o	o		●			o	o	o				o	●
C369	o				o	●	o			●		o	o					●			o	o	o	o	o	o	●	o	o	●	
C372			o		o	o	o	●	o		o	o	o	o		o			o	o	o	o	o	o		o	o	o		●	●
C379	o	●	o	o	●	o	●		o	o		o	o	o	o	o		o	●	o	o	o	o		o	o	o	o			o
C385			●	o	●		o	●	o	o		●	o	o	●	o			●	●			●	●	o	o	o		●	o	
C399			●	o	o	o			o			o	o	o							o		o		o	o			o	o	●
C400			o	o	o	o	o	●	o	o		o		o	o		o	o	o			o	o	o	o	●	o		o		
C401	o	●	o		o	o	o		o	o				o	o				o		o	o	●		o	o	●		o	●	●
C403	o	o	o				o	●		o			o	o	o		o	o	o		o	o			o	o	o		o	o	o
C404	o			o	●	o	o	●	o	●	o		o	o				o	o				o	●	o	o	●		o	o	o
C405				o		o		o		o	o	o		o		o	o		o	o	o	o	o	o	o	o	o	o	o	o	
C407			o	o	o	o	o		o	●		o	o	o		o	o		o	o	o		o	o	o	o	o	o	●		
C408			o		o	o		o			●	●			●	o		●	●	o		●	o		o		o		o	●	●
C409		o		o	o		o	o	●		●	o	o			o	●				o	o		o	●	o	●		o	●	●
C411	●		o	●		●		o	o			o		o	o	o	o		o		o	o	o		●	o	o	●	o	o	o
C412	●	●	●	o		o		●				●	●		o	o	o				●		o	●	●	o	o	o	●	●	
C415		o	o	o		o	o		o			o	o	o		o					o			o	o	o	o		o	●	o
C419	●			o		o	o		o	o		o		o	o	o	o		o		o	o	●	o	●	o	o		o		●
C421	o			o	●			o	o			●	o	●		o	●		●	●					o	●		●	●	o	
C422	o		o				o	o		o	o					o	o		o	●	o	o		●	o	o			o	o	
C437			o	o		o	o	o	o	o	o	o			o	o	o		o	o	o	●			o	o	o		o			
C438	o	●	o	o		o	o	o		o	o	o			o	o	o		o		o	o			o	o	o		o	o	●
C440				o	o	o	o			o	o	o	o		o	o		o	o		o	o			o	o		o	o		
C444	●		●		o	o	o		o			o						o	o	o	o	o		●	o	o			o	●	o
C445	o		●	o			o	o	o	●	o	●			o	o	●	o	o	o			●	o	o	o					●
C452				o		o		o	o	o		o	●		o	o		●	o		o	o		●	o	o					●
C455					●	●	o	o	o	●	o	o	o		●			o	o		o	●		o	●	o				o	o

Figure 4. 1 LOH pattern on chromosome 1 in cervical cancer

•: Informative case with LOH o: Informative case without LOH Blank: Uninformative case.

Table 4.1 Frequency of LOH at 31 loci on chromosome 1 in 100 cervical carcinomas

Name of primer	Locus symbol	Map	No. of informative tumours	No. of cases with LOH (%)
AFMa210xg9	**D1S2663**	**1p36.3**	**46**	**16 (35%)**
AFMa127zc9	D1S507	1p36.3	84	16 (19%)
AFM344we9	D1S2845	1p36.2	77	12 (16%)
AFMa162zc9	D1S2647	1p36.1	59	15 (25%)
AFM200wh8	D1S2722	1p36.1	40	8 (20%)
AFMa048yh1	D1S2864	1p34.3	52	14 (27%)
AFMa190zc5	D1S2656	1p34.3	60	17 (28%)
AFMa140yh1	D1S2638	1p34.1	59	10 (17%)
AFMa082wb5	D1S2890	1p33	64	14 (22%)
AFMa060yh9	D1S2882	1p33	65	5 (8%)
AFM303vc5	**D1S2829**	**1p31**	**46**	**20 (44%)**
AFM350tg9	D1S2849	1p31	62	11 (18%)
AFM113xf6	D1S206	1p21	82	20 (25%)
AFM207yh6	D1S2346	1p13	92	25 (27%)
AFMa133ye5	D1S2635	1q12	46	10 (22%)
AFMa057ze5	D1S2878	1q21	67	15 (22%)
AFMb359xf5	D1S2799	1q21	53	15 (28%)
AFMa285zd5	D1S2691	1q24	50	6 (12%)
AFM205ag1	D1S238	1q25	95	16 (17%)
AFMb309xe1	**D1S2757**	**1q25**	**55**	**17 (31%)**
AFM031xd12	D1S412	1q25	78	5 (6%)
AFMa190xd5	D1S2655	1q25	63	13 (21%)
AFM095ta5	D1S202	1q31	85	10 (12%)
AFMa303wh5	D1S2703	1q31	72	11 (15%)
AFMa290xd1	D1S2692	1q32	73	10 (14%)
AFM321xe5	D1S2833	1q41	76	16 (21%)
AFM164yg1	D1S222	1q42	60	11 (18%)
AFM123yc7	D1S461	1q42	59	10 (17%)
AFMb349xb	D1S2785	1q42	69	11 (16%)
76.10 76.11	**D1S76**	**1P36-35**	**80**	**34 (43%)**
80.3 80.4	**D1S80**	**1P36-35**	**77**	**35 (45%)**

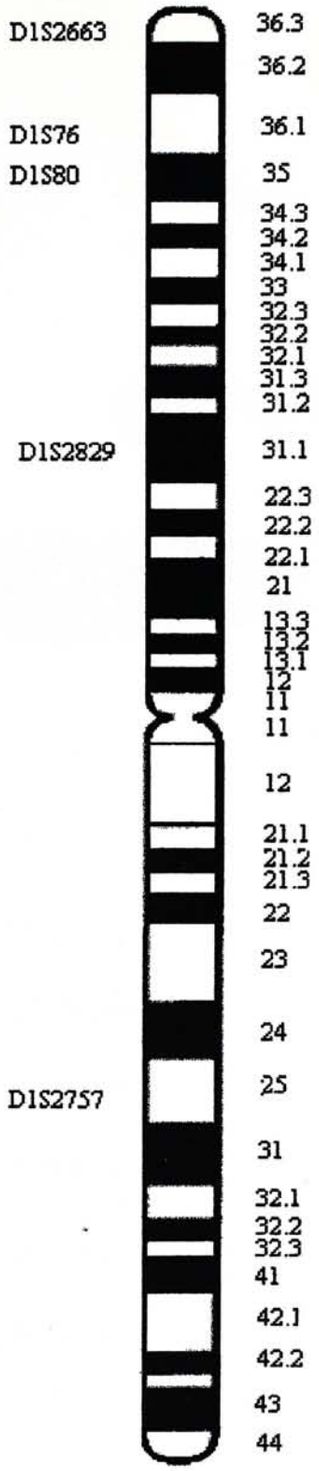


Fig 4.2 The location of makers D1S76, D1S80, D1S2829, D1S2663, D1S2757. LOH is frequently detected at these loci in cervical cancer.

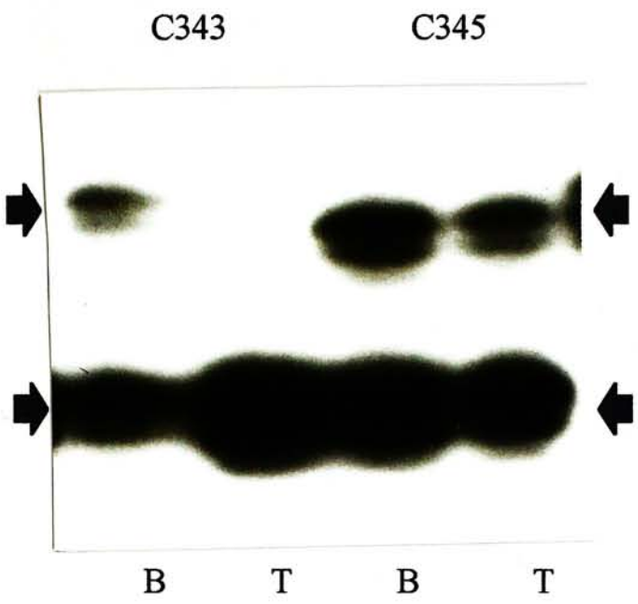


Figure 4.3 LOH analysis at locus D1S2845 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C343 shows loss of the higher allele inferred as LOH, while case C345 displays retention of heterozygosity at this locus. B, blood DNA; T, tumour tissue DNA.

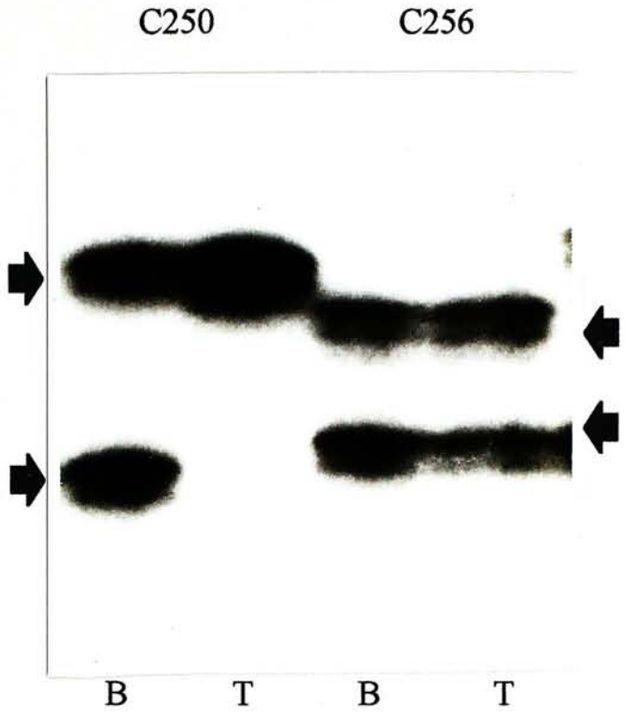


Figure 4.4 LOH analysis at locus D1S2878 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C250 shows loss of the lower allele inferred as LOH, while case C256 displays retention of heterozygosity at this locus. B, blood DNA; T, tumour tissue DNA.

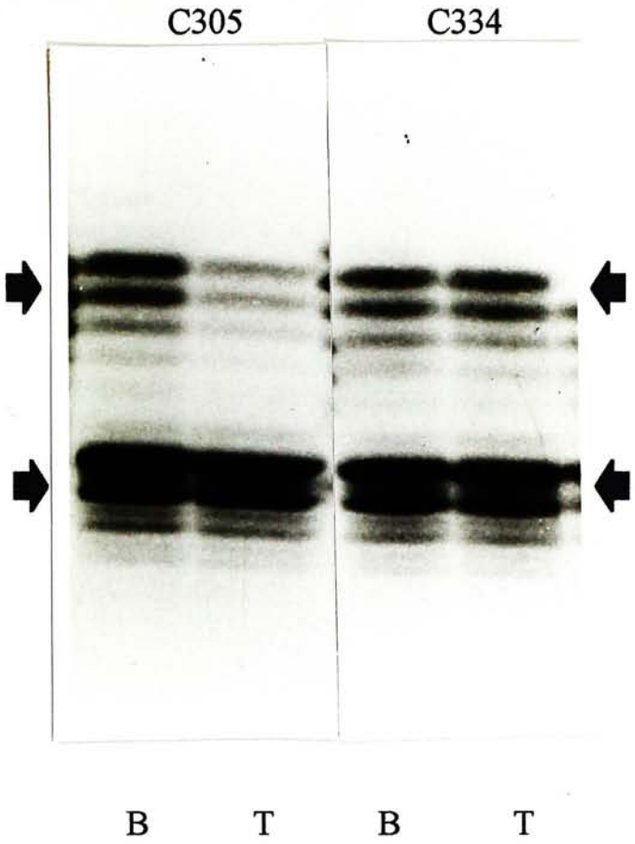
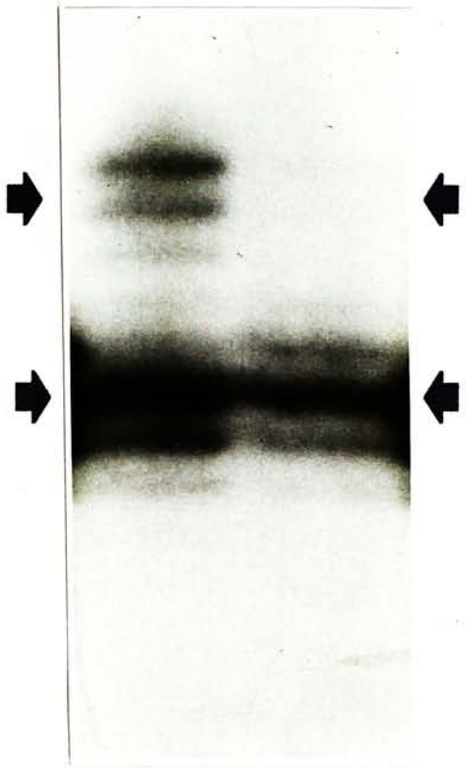


Figure 4.5 LOH analysis at locus D1S206 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C305 shows very faint higher allele inferred as LOH also, while case C334 displays retention of heterozygosity at this locus. B, blood DNA; T, tumour tissue DNA.

C265



B T

Figure 4.6 LOH analysis at locus D1S2785 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C265 shows loss of the higher allele inferred as LOH. B, blood DNA; T, tumour tissue DNA.

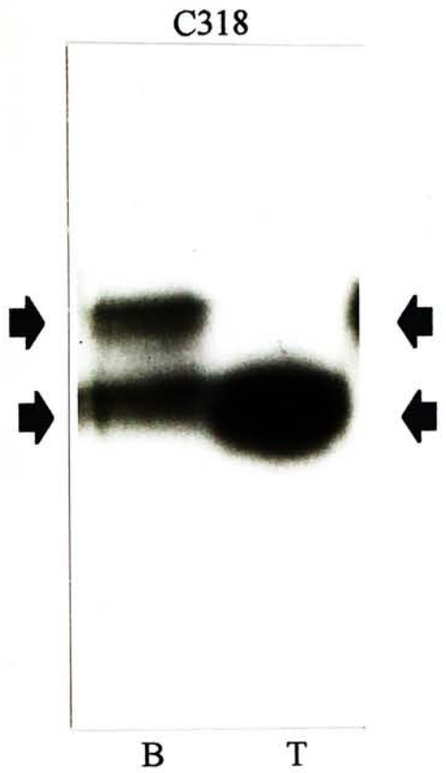


Figure 4.7 LOH analysis at locus D1S2757 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C318 shows loss of the higher allele inferred as LOH. B, blood DNA; T, tumour tissue DNA.

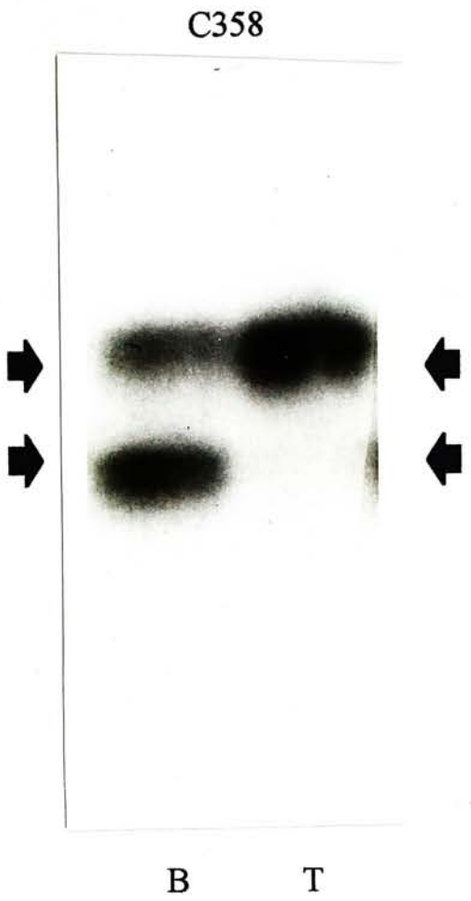


Figure 4.8 LOH analysis at locus D1S2635 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C358 shows loss of the lower allele inferred as LOH. B, blood DNA; T, tumour tissue DNA.

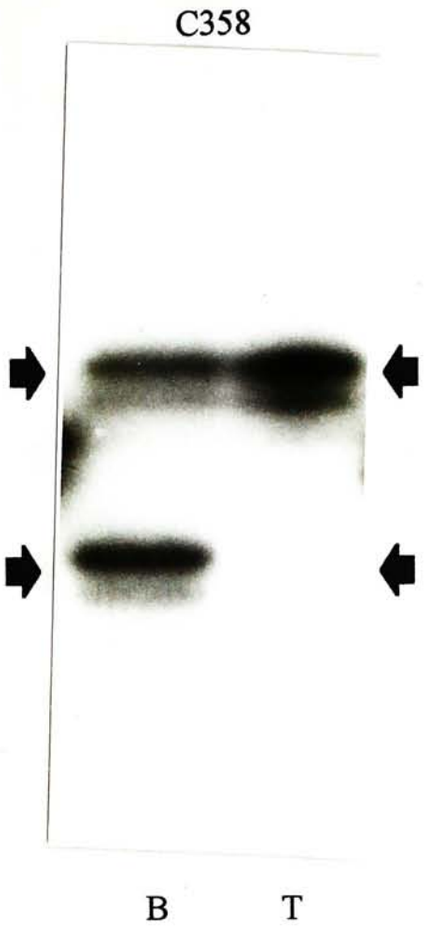


Figure 4.9 LOH analysis at locus D1S412 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C358 shows loss of the lower allele inferred as LOH. B, blood DNA; T, tumour tissue DNA.

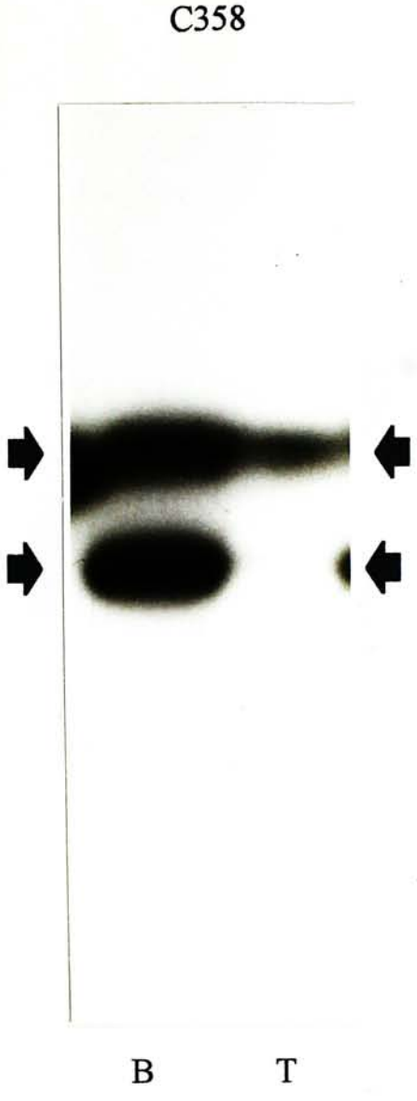


Figure 4.10 LOH analysis at locus D1S222 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C358 shows loss of the lower allele inferred as LOH. B, blood DNA; T, tumour tissue DNA.

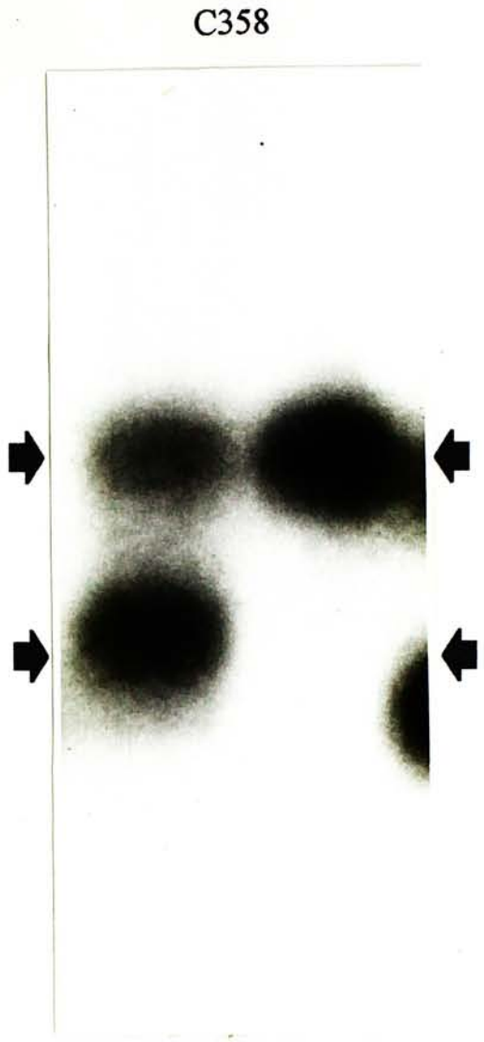


Figure 4.11 LOH analysis at locus D1S2829 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C358 shows loss of the lower allele inferred as LOH. B, blood DNA; T, tumour tissue DNA.

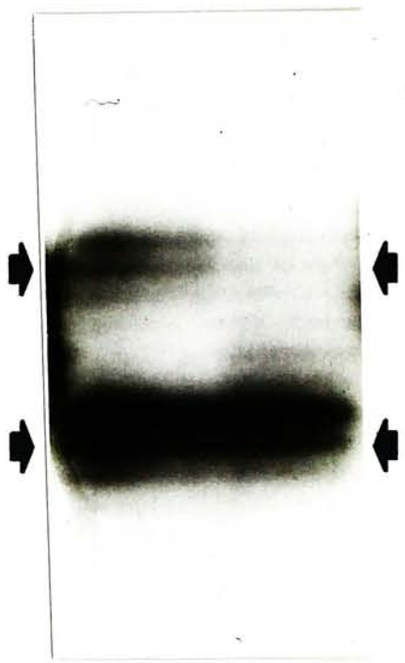
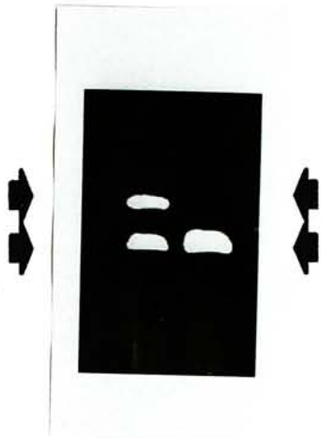


Figure 4.12 LOH analysis at locus D1S2703 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C283 shows very faint higher allele inferred as LOH. B, blood DNA; T, tumour tissue DNA.

C232



B T

Figure 4.13 LOH analysis at locus D1S76 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C232 shows loss of the higher allele inferred as LOH. B, blood DNA; T, tumour tissue DNA.

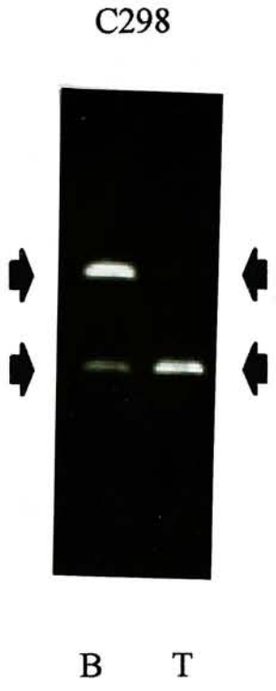


Figure 4.14 LOH analysis at locus D1S80 in cervical cancer. Arrowheads, positions of the alleles. In addition to main allele(s), faster-migrating stutter brands and more slowly migrating conformational bands are present. Case C298 shows loss of the higher allele inferred as LOH also. B, blood DNA; T, tumour tissue DNA.

4.2 LOH and age in cervical cancer

The age range of the patients with cervical cancer in this study was from 23 to 83 years old with the mean age of 56 years. The patients were classified into two groups: (1) < 40 years, and (2) ≥40 years. Table 4.2 shows prevalence of LOH at various loci on chromosome 1 in two age groups of cervical cancer.

Comparison of LOH in cervical cancer with age revealed no statistical correlation ($p > 0.05$).

Table 4.2 Correlation of LOH on chromosome 1 with age in cervical cancer

Locus symbol	No. of case with LOH /No. of Informative case (%)		*P* value
	<40	≥40	
D1S2890	2/9 (22%)	12/55 (22%)	>0.05
D1S2829	3/5 (60%)	17/41 (41%)	>0.05
D1S2757	2/7 (29%)	15/48 (31%)	>0.05
D1S2703	1/11 (9%)	10/61 (16%)	>0.05
D1S2635	1/7 (14%)	9/39 (23%)	>0.05
D1S2878	1/10 (10%)	14/57 (25%)	>0.05
D1S2655	1/7 (14%)	12/56 (21%)	>0.05
D1S2833	2/8 (25%)	14/68 (21%)	>0.05
D1S2882	2/9 (22%)	3/56 (5%)	>0.05
D1S2849	1/6 (17%)	10/56 (18%)	>0.05
D1S2647	0/2 (0%)	15/57 (26%)	>0.05
D1S2845	1/10 (10%)	11/67 (16%)	>0.05
D1S461	1/5 (20%)	9/54 (17%)	>0.05
D1S202	3/12 (25%)	7/73 (10%)	>0.05
D1S222	1/15 (7%)	10/55 (18%)	>0.05
D1S412	1/9 (11%)	4/69 (6%)	>0.05
D1S2799	1/8 (13%)	14/45 (31%)	>0.05
D1S2663	1/8 (13%)	15/38 (39%)	>0.05
D1S2346	3/11(27%)	22/81 (27%)	>0.05
D1S2722	1/3 (33%)	7/37 (19%)	>0.05
D1S2785	2/8 (25%)	9/61 (15%)	>0.05
D1S2638	0/5 (0%)	9/54 (17%)	>0.05
D1S2864	2/5 (40%)	12/47 (26%)	>0.05
D1S2656	1/5 (20%)	16/55 (29%)	>0.05
D1S238	3/11 (27%)	13/84 (15%)	>0.05
D1S507	2/9 (22%)	14/75 (19%)	>0.05
D1S206	1/10 (10%)	19/72 (26%)	>0.05
D1S2691	0/6 (0%)	6/44 (14%)	>0.05
D1S2692	0/10 (0%)	10/63 (16%)	>0.05
D1S76	5/10 (50%)	32/67 (48%)	>0.05
D1S80	4/8 (50%)	39/69 (57%)	>0.05

4.3 LOH and pathologic grade in cervical cancer

According to WHO criteria, the histological grades of cervical cancer were divided into categories 1, 2 and 3.

In the study, 18 cases were grade 1, 58 cases were grade 2 and 24 cases were grade 3. Table 4.3 shows the correlation of LOH on chromosome 1 with pathologic grades in cervical cancer.

At locus of D1S2878 ($p < 0.05$), the frequency of LOH in grade 1 was 7%, in grade 2 was 19% and in grade 3 was 44%. The difference was statistically significant ($p < 0.05$).

However, there were no significant correlation of LOH with pathologic grade of the cervical cancers at any of other loci on chromosome 1.

Table 4.3 Correlation of LOH on chromosome 1 with pathologic grades in cervical cancer

Locus symbol	No. of case with LOH /No. of Informative case(%)			*P* value
	Grade 1	Grade 2	Grade3	
D1S2890	4/12(33%)	7/34(21%)	3/17(18%)	>0.05
D1S2829	5/9 (56%)	9/24(38%)	6/13(46%)	>0.05
D1S2757	5/11(45%)	8/33(24%)	4/11(36%)	>0.05
D1S2703	2/15(13%)	6/44(14%)	3/13(23%)	>0.05
D1S2635	2/8(25%)	6/28(21%)	2/9(22%)	>0.05
D1S2878	**1/15(7%)**	**7/36(19%)**	**7/16(44%)**	**<0.05**
D1S2655	1/10(10%)	7/36(19%)	5/17(29%)	>0.05
D1S2833	3/13(23%)	9/46(20%)	4/17(24%)	>0.05
D1S2882	1/14(7%)	4/36(11%)	0/15(0%)	>0.05
D1S2849	1/9(11%)	7/37(19%)	3/16(19%)	>0.05
D1S2647	3/8(38%)	9/35(26%)	3/16(19%)	>0.05
D1S2845	4/14(29%)	5/43(12%)	3/20(15%)	>0.05
D1S461	3/11(27%)	5/35(14%)	2/13(15%)	>0.05
D1S202	3/14(21%)	5/52(10%)	2/19(11%)	>0.05
D1S222	2/13(15%)	5/35(14%)	4/12(33%)	>0.05
D1S412	0/16(0%)	2/44(5%)	3/18(17%)	>0.05
D1S2799	1/12(8%)	12/32(38%)	2/9(22%)	>0.05
D1S2663	1/8(13%)	9/23(39%)	6/15(40%)	>0.05
D1S2346	4/17(24%)	17/52(%33%)	4/23(17%)	>0.05
D1S2722	1/8(13%)	4/21(19%)	3/11(27%)	>0.05
D1S2785	1/14(7%)	8/40(20%)	2/15(13%)	>0.05
D1S2638	2/9(22%)	6/36(17%)	2/14(14%)	>0.05
D1S2864	4/8(50%)	7/33(21%)	3/11(27%)	>0.05
D1S2656	4/8(50%)	8/37(22%)	5/15(33%)	>0.05
D1S238	5/17(29%)	7/57(12%)	4/21(19%)	>0.05
D1S507	2/15(13%)	9/48(19%)	5/21(24%)	>0.05
D1S206	3/15(20%)	12/50(24%)	4/17(24%)	>0.05
D1S2691	1/9(11%)	4/27(15%)	1/14(7%)	>0.05
D1S2692	2/16(13%)	6/43(14%)	2/14(14%)	>0.05
D1S76	3/12(25%)	22/49(45%)	9/19(47%)	>0.05
D1S80	6/9(67%)	18/46(39%)	10/21(48%)	>0.05

4.4 LOH and clinical stage in cervical cancer

Clinical staging was determined by the criteria of the International Federation of Gynecology and Obstetrics(FIGO). 68 cases with LOH were classified into stage I/II and 32 cases in stage III/IV. Table 4.4 shows analysis of relationship between LOH and clinical stage.

The correlation of LOH with clinical stage at loci D1S2829, D1S2757, D1S2647 and D1S2663 was significant ($p < 0.01$ - 0.05). The frequency of LOH increased in the late stage. There was no significant difference in the incidence of LOH between early and late stage of cervical cancer in other markers studied ($P > 0.05$).

Table 4.4 Correlation of LOH on chromosome 1 with clinical stage in cervical cancer

Locus symbol	No. of case with LOH /No. of Informative case(%)		*P* value
	Stage I -II	Stage III-IV	
D1S2890	10/45(22%)	4/19(21%)	>0.05
D1S2829	**6/24(25%)**	**14/22(64%)**	**<0.05**
D1S2757	**6/33(18%)**	**11/22(50%)**	**<0.05**
D1S2703	5/48(10%)	6/24(25%)	>0.05
D1S2635	6/34(18%)	4/12(33%)	>0.05
D1S2878	7/43(16%)	7/24(29%)	>0.05
D1S2655	9/42(21%)	4/21(19%)	>0.05
D1S2833	8/50(16%)	8/26(31%)	>0.05
D1S2882	4/48(8%)	1/17(6%)	>0.05
D1S2849	9/44(20%)	2/18(11%)	>0.05
D1S2647	**5/39(13%)**	**10/20(50%)**	**<0.01**
D1S2845	30/55(55%)	9/22(41%)	>0.05
D1S461	3/40(8%)	7/19(37%)	>0.05
D1S202	7/58(12%)	3/27(11%)	>0.05
D1S222	4/37(11%)	7/23(30%)	>0.05
D1S412	4/50(8%)	1/28(4%)	>0.05
D1S2799	10/39(26%)	5/14(36%)	>0.05
D1S2663	**5/29(17%)**	**11/17(65%)**	**<0.01**
D1S2346	18/62(29%)	7/30(23%)	>0.05
D1S2722	7/27(26%)	1/13(8%)	>0.05
D1S2785	4/46(9%)	7/25(28%)	>0.05
D1S2638	6/42(14%)	4/17(24%)	>0.05
D1S2864	8/37(22%)	6/15(40%)	>0.05
D1S2656	9/39(23%)	7/21(33%)	>0.05
D1S238	13/64(20%)	3/31(10%)	>0.05
D1S507	13/60(22%)	3/24(13%)	>0.05
D1S206	11/53(21%)	9/29(31%)	>0.05
D1S2691	4/33(12%)	2/16(13%)	>0.05
D1S2692	6/51(12%)	4/23(17%)	>0.05
D1S76	20/55(36%)	14/25(56%)	>0.05
D1S80	19/50(38%)	16/27(59%)	>0.05

4.5 LOH and clinical status in cervical cancer

There are three groups of clinical status in cervical cancer such as:

(1) alive with no evidence of disease (NED)

(2) alive with disease (AWD)

(3) died of disease (DOD) .

The correlation of LOH with clinical status was analysed as shown in Table 4.5. The frequency of LOH at loci D1S2829, D1S2757, D1S2647, D1S2845, D1S461 and D1S2663 in the patients who had died of their disease or alive with evidence of disease is higher than that in those without evidence of disease. The differences were statistically significant ($p < 0.01$-0.05).

Table 4.5 Correlation of LOH on chromosome 1 with clinical status in cervical cancer

Locus symbol	No. of case with LOH /No. of Informative case (%)			*P* value
	NED*	AWD*	DOD*	
D1S2829	11/46 (24%)	3/16 (19%)	0/2 (0%)	>0.05
D1S2829	**8/26 (31%)**	**11/11(100%)**	**1/2 (50%)**	**<0.01**
D1S2757	**7/35 (20%)**	**9/19 (47%)**	**1/1 (100%)**	**<0.05**
D1S2703	**4/50 (8%)**	**7/20 (35%)**	**0/2 (0%)**	**<0.05**
D1S2635	**4/34 (12%)**	**6/11 (55%)**	**0/1 (0%)**	**<0.05**
D1S2878	11/46 (24%)	4/19 (21%)	0/2 (0%)	>0.05
D1S2655	11/44 (25%)	2/16 (13%)	0/3 (0%)	>0.05
D1S2833	8/51 (16%)	7/22 (32%)	1/3 (33%)	>0.05
D1S2882	4/49 (8%)	1/15 (7%)	0/1 (0%)	>0.05
D1S2849	10/46 (22%)	1/15 (7%)	0/1 (0%)	>0.05
D1S2647	**6/40 (15%)**	**7/17 (41%)**	**2/2 (100%)**	**<0.01**
D1S2845	**4/56 (7%)**	**7/18 (39%)**	**1/3 (33%)**	**<0.01**
D1S461	**5/43 (12%)**	**4/15 (27%)**	**1/1 (100%)**	**<0.05**
D1S202	7/61 (11%)	3/23 (13%)	0/1 (0%)	>0.05
D1S222	5/39 (13%)	6/18 (33%)	0/3 (0%)	>0.05
D1S412	5/53 (9%)	0/22 (0%)	0/3 (0%)	>0.05
D1S2799	10/40 (25%)	4/11 (36%)	1/2 (50%)	>0.05
D1S2663	**6/31 (19%)**	**9/13 (69%)**	**1/2 (50%)**	**<0.01**
D1S2346	16/65 (25%)	9/25 (36%)	0/2 (0%)	>0.05
D1S2722	5/27 (19%)	3/12 (25%)	0/1 (0%)	>0.05
D1S2785	6/48 (13%)	4/18 (22%)	1/3 (33%)	>0.05
D1S2638	6/44 (14%)	4/13 (31%)	0/2 (0%)	>0.05
D1S2864	8/38 (21%)	6/13 (46%)	0/1 (0%)	>0.05
D1S2656	10/42 (24%)	7/15 (47%)	0/3 (0%)	>0.05
D1S238	13/66 (20%)	2/26 (8%)	1/3 (33%)	>0.05
D1S507	13/61 (21%)	2/20 (10%)	1/3 (33%)	>0.05
D1S206	12/56 (21%)	7/23 (30%)	1/2 (50%)	>0.05
D1S2691	4/37 (11%)	2/12 (17%)	0/1 (0%)	>0.05
D1S2692	6/51 (12%)	4/10 (40%)	0/2 (0%)	>0.05
D1S76	**21/56 (38%)**	**13/21 (62%)**	**0/3 (0%)**	**<0.05**
D1S80	22/53 (42%)	11/21 (52%)	2/3 (67%)	>0.05

*NED, alive with no evidence of disease
*AWD, alive with disease
*DOD, died of disease

Chapter Five

Discussion

Genetics as we know it today is largely the result of research done during this century. All genetic variation originates from the process known as mutation , which is defined as a change in DNA sequences. Mutations can affect either somatic or germline cells. Mutations in somatic cells can lead to cancer (Jorde *et al.*, 1995).

The genetic changes (mutations) that are known to cause or contribute to neoplastic growth occur within genes belonging to two major gene classes, the proto-oncogenes and the tumour suppressor genes. The proteins encoded by these genes perform opposing functions during normal homeostasis, the proto-oncogenes stimulate cell proliferation and the tumour suppressors inhibit cell proliferation. The balance is altered if a proto-oncogene is activated to an oncogene, or if a tumour suppressor gene is inactivated, either of which can result in abnormal growth stimulation (Rothe *et al.*, 1997).

Some mutations consist of an alteration of the number or structure of chromosomes in a cell. These major chromosome abnormalities can be observed microscopically. However, mutation affecting only single gene, or deletions of one or more base pairs are not microscopically observable. The restriction fragment length polymorphisms (RFLP) and variable number of tandem repeats (VNTR) approaches, which typically depend on Southern blotting and cloning procedures, have been useful in many applications. However, they suffer from

certain limitations. Cloning is time-consuming, often requiring a week or more of laboratory time. In addition, the standard Southern blotting approach requires relatively large amounts of purified DNA, usually several micrograms (up to 1 ml of fresh blood would be needed to produce this much DNA). A newer approach to make copies of DNA, the polymerase chain reaction (PCR), has made the detection of genetic variation at the DNA level much more efficient.

5.1 Microsatellite markers

Microsatellites are composed of one to six bases of repeated DNA sequence (Eshleman *et al.*, 1995) that are usually repeated 15-30 times and distributed throughout the genome. Sometimes a distinction is made between 2 bp repeat microsatellite and 3-5 bp short tandem repeats (Lothe *et al.*, 1997). The most common repeat is (A)n and the second most common is (CA)n, where n=10 to 60 (eg, [CA]3=CACACA) (Weber *et al.*, 1989; Litt *et al.*, 1989; Hearne *et al.*, 1992; Eshleman *et al.*, 1995). They are generally considered noncoding because they are most commonly present in introns and between genes; however, tri-nucleotide repeats can be coding in some cases (Eshleman *et al.*, 1995). The dinucleotide repeats are estimated to be the most frequently occurring microsatellite, with an incidence of at least 1 every 100,000bp (Lothe *et al.*, 1997). Because there are an estimated 10,000 to 50,000 copies of the CA repeat randomly distributed throughout the genome and as the repeats are highly polymorphic, they are used extensively for "DNA fingerprinting," paternity testing, linkage mapping, study of chromosomal loss in tumours, and for distinguishing donor versus recipient cells following bone marrow transplantation.

Despite their high polymorphism between individuals, the pattern with which an individual is born is fixed for life, and this pattern can therefore be found in any tissue or tumour from the individual (Eshleman *et al.*, 1995). Locus specific fragments can be amplified by PCR, by using the short sequences flanking the repeats as primers. This locus specificity, and the fact that the two parental alleles are often of different lengths because of variable number of repeats between them, makes these polymorphic markers suitable for a wide range of analyses. Since the initial reports of microsatellite instability in colorectal carcinomas, such markers mapping to all human chromosomes have been used to analyse numerous human tumours from various tissues (Lothe *et al.*, 1997).

The use of microsatellite markers has permitted the construction of new genetic maps of the human genome as well as those of other mammals (Dietrich *et al.*, 1992; Weissenbach *et al.*, 1992; Serikawa *et al.*, 1992; Gyapay *et al.*, 1994). These maps can be used to map any Mendelian trait. A new genetic linkage map contained a total of 2,066 (AC)n short tandem repeats 60% of which show a heterozygosity of over 0.7. Statistical linkage analysis based on the genotyping of eight large CEPH families placed these markers in the 23 linkage groups. The map includes 1,266 intervals and spans a total distance of 3690 centiMorgans (cM). About 56% of this map is at a distance of 1 cM or less from one of its markers (Gyapay *et al.*, 1994).

5.2 PCR condition

Essentially PCR is an artificial means of replicating a short DNA

sequence (several kilobases or less) quickly, so that millions of copies of the sequence are made (Jorde *et al.*, 1995). Because of its speed and ease of use, this technique is now widely used for assessing genetic variation, diagnosing genetic diseases and for forensic purposes. The extreme sensitivity of PCR makes it susceptible to contamination and a number of precautions are commonly taken to guard against contamination.

In this study we used Taqstart antibody in materials to enhance the specificity and sensitivity of PCR amplification. Taqstart antibody is a neutralising monoclonal antibody to *Taq* DNA polymerase. It can block polymerase activity during set-up of the PCR reactions at room temperatures (20-22°C). The inhibition of *Taq* DNA polymerase is completely reversed when the temperature is raised above 70°C. At the first template denaturation step in thermal cycling, the enzyme-antibody complex dissociates and the Taqstart antibody is rendered non-functional. At the same time, the activity of *Taq* DNA polymerase is restored, and the enzyme functions normally during the course of the PCR. In many cases Taqstart antibody also prevents generation of non-specific amplification products and primer-dimer artefacts.

The use of fluorescently labelled nucleotides (F-dUTP) and isotope in the PCR for genetic typing of microsatellite polymorphisms has been investigated. The use of F-dUTP is considered as rapid and inexpensive methods for analyses of genetic markers, such as microsatellite polymorphisms which is essential to studies of genetic disease and for mapping of cancer genes. The main

disadvantages of the incorporation method are the necessity for purification before the gel analysis and the tendency to yield broader peaks, possibly due to nonquantitative incorporation of label, incomplete denaturation of the secondary structures of unequal distribution of fluorophores between the two strands (Patril *et al.*, 1996). Many studies use [α-32p]dCTP (Busby-Earle *et al.*, 1993; Mitra *et al.*, 1994; Ezaki *et al.*, 1996; Grace *et al.*, 1992) in their research. It is convenient, does not require labelling, has a higher sensitivity and provides clean result. On the other hand, it needs to be fixed it in 5% acetic acid, 5% methanol and large amount of isotope for each reaction, it is relatively expensive than [γ-32p]ATP.

In my study we used [γ-32p]ATP. This technique is highly sensitive and economical and only needs 0.5 μl (10 mCi/ml) for 50 reactions. It is not necessary to fix it in 5% acetic acid and 5% methanol. It only needs to expose to x-ray film for 12-24 hours and labelling time.

5.3 LOH in cervical cancer

Functional inactivation of a tumour suppressor genes often involves deletion of the normal allele to unmask the mutated allele. Therefore, chromosomal regions that are frequently lost are thought to harbour putative suppressors. LOH analyses are used to detect the chromosomal deletion in tumour cell when the constitutional DNA has two different alleles at a locus. Recent studies based on allelic deletions in various types of tumours have implied the presence of a putative tumour-suppressor gene(s) on chromosome 1. These

results have been reported in colorectal cancers (Leister *et al.*, 1990; Bardi *et al.*, 1993), hepatocellular carcinomas (Simon *et al.*, 1991; Yeh *et al.*, 1994), neuroblastomas (Fong *et al.*, 1989; Wetih et al., 1989; Schleiermacher *et al.*, 1994; Takeda *et al.*, 1994), phaeochromocytomas and medullary thyroid carcinomas (Mathew *et al.*, 1987; Moley *et al.*, 1992) and breast cancers (Biecge *et al.*, 1993; Dracopoli *et* al., 1994; Loupart *et al.*, 1995). As the commonly deleted region observed in gastric cancers overlaps with the commonly deleted regions reported in various other tissues (Mathew *et al.*, 1987; Fong *et al.*, 1989; Leister *et al.*, 1990; Moley *et al.*, 1992), inactivation of the same unidentified tumour-suppressor gene(s) on the short arm of chromosome 1 may be associated with more than one type of cancer (Tungekar *et al.*, 1996).

We employed 31 markers on chromosome 1 to find allelic loss. LOH was detected in 96 of 100 (96%) cases of cervical cancer. The number of tumours informative for each locus ranged from 40% to 95%. LOH at two or more than two loci was detected in 88 of 95 (93%) informative cervical carcinomas, while 8 of 95 cases (8.4%) had LOH detected only at one locus, suggesting that chromosomal aberrations causing LOH on chromosome 1 are relatively common in cervical cancer. In our extensively studied cases, the highest incidence of LOH was at locus D1S80 (45%). The locus of D1S2829 (44%) showed the second most frequent area of loss. D1S76 (43%) also showed frequent LOH. The rate at loci D1S2663 and D1S2757 was 35% and 31% respectively. These five loci showed high frequency of LOH. Another 12 loci were involved in LOH in 20-28% of the informative tumours. The finding of 31-45% LOH at the loci

D1S2829 (1p31), D1S76 (1p36-35), D1S80 (1p36-35), D1S2663 (1p36.3) and D1S2757 (1q25) strongly suggests that there are the sites of one or more tumour suppressor genes which play an important role in the development or progression of cervical carcinoma. Cervical cancer-specific tumour suppressor gene(s) may be located in chromosome 1p31, 1p36-35 and 1q25.

Nagai *et al.* (1995) and Munn *et al.* (1995) by analysing highly polymorphic microsatellite loci in breast cancer, they found four common regions were defined with 1p LOH, two of which were 1p36 and 1p31.

Another most detailed picture to date has been presented by Praml *et al.* (1995) who employed 33 microsatellite markers on 1p to find allelic loss in 84% of the cases in colorectal cancer. The identification of independent interstitial deletions indicated that 1p harbour more than one genetic locus related to colorectal cancer. The valuation of data suggested the existence of three regions that may contain genetic information. All three regions are within bands 1p34.2-pter (Schwab *et al.*, 1996).

Our result indicates frequent LOH at loci D1S2656 and D1S2663, which mapped at 1p34.3 (28%) and 1p36.3 (35%). Both D1S76 and D1S80 loci used for the present LOH-PCR determination have been genetically located close to the telomeric D1Z2 in 1p36 band (Dracopoli *et al.*, 1991; Wong *et al.*, 1993).

Loss of heterozygosity studies confirmed a high frequency (41%) of

allelic loss at the subtelomeric locus D1Z2 in 1p36 in breast (Genuardi *et al.*, 1989; Schwab *et al.*, 1996). These loci were thus expected to be involved in most of the 1p deletions tumours.

Several studies have placed a shortest region of overlap for the 1p deletions to chromosomal band 1p36. Such as breast carcinoma (Genuardi *et al.*, 1989; Schwab *et al.*, 1996), neutroblastoma (Peter *et al.*, 1992), suggesting that a tumour suppressor gene may be located here. It remains to be determined whether the same or different genes located within these segments are involved in the different cancer types.

In our study, cervical carcinomas show 40-45% allelic loss at the locus D1S76 and D1S80. The result is similar to the two original studies employed two independent sets of molecular probes. It is therefore concluded that the chromosomal region which is most consistently involved spans 1p36.3 to 1p35 (Fong *et al.*, 1989; Schwab *et al.*, 1996).

Of particular significance might be the identification of a constitutional interstitial deletion affecting 1p36 in a patient with neuroblastoma (Biegel *et al.*, 1993; Schwab *et al.*, 1996). The systematic use of highly polymorphic microsatellites has narrowed down the region of consistent deletion in tumours within 1p36 to 1p36.2-1p36.3. A region deleted in the great majority of all analysed tumours is bounded distally by D1S80 (Takeda *et al.*, 1994; Martinson *et al.*, 1995; White *et al.*, 1995; Schwab *et al.*, 1996). Few LOH studies appear to

indicate the existence of more than one suppressor locus in 1p. First indications came from a study by Takeda *et al.* (1994) who found 1p LOH for 21 of 104 tumours analysed. Seven of these showed an interstitial deletion, which encompassed a small region in 1p36. Cryns *et al* examined 25 tumours with polymorphic probe from different chromosome, the highest frequency of LOH (40%) was observed at 1p32-36 for both loci D1S80 and D1S57.

Reported by Michael *et al* (1996), chromosome arm 1q has LOH in 20-33% of the tumours. Our results shows LOH in 1q ranges from 6% to 31%. Mitra *et al* (1994) examined 53 primary cervical carcinomas, they found LOH on 1p is 2% and 1p32-p35 is 0%, and they revealed frequent LOH at 1q31-q32 is 26%. Rader *et al.* (1996) found the frequency of LOH in 1p and 1q between 0-8%. Our studies show the frequent range from 8-44% in 1p. The discrepancy may be due to the difference number of informative cases in their study and /or the difference in the map location of the probes.

Our results indicate that a significant number of invasive cervical cancers have lost specific chromosomal regions, thereby suggesting that genes involved in the cell cycle regulation or the suppression of tumour development are located in these regions.

5.4 Correlation of LOH with clinico-pathologic characteristics of cervical cancer

Cytogenetic analyses have revealed chromosome 1 as the predominant

target for alterations in cervical carcinomas (Sreekantaiah *et al.*, 1988), particularly bands p36-35 (Zimonjic *et al.*, 1995), and it has been suggested that changes in 1p represent a primary alteration in cervical neoplasia (Sreekantaiah *et al.*, 1991). They have indicated a correlation of 1p deletion with bad prognosis (Christiansen and Lampert *et al.*, 1988; Hayashi *et al.*, 1989). Caron *et al.* (1993) reported that allelic loss of 1p identifies patients at high risk of an unfavourable outcome. It is obvious, though, that roughly one third of the patients with 1p loss are long term survivors, thus making 1p loss an unreliable marker and raising the question how to apply this information to the benefit of the patient. A correlation has not been seen by Gehring *et al.* (1995) or Maris *et al.* (1995) and was not confirmed in a more recent evaluation by Christiansen *et al.* (1995), suggesting that the value of 1p loss as a marker of prognosis is at least unclear.

Correlating stage, histological types and other histopathological factors with LOH at a certain chromosomal locus may help to identity an independent prognostic factors and establish a potential link between areas of chromosome loss and specific properties of tumours (Huettner *et al.*, 1998).

We correlate our findings with age, histologic grade, clinical status and clinical stage of the cancer. As the histological types of our cases are mostly squamous cell carcinomas, we do not correlate histological types of cervical cancer with LOH.

5.4.1 LOH and age

We find that tumours in women less than 40 years exhibit an overall higher frequency of LOH, compared to that in patients over 40 years of age, but the difference is statistically insignificant ($p > 0.05$).

5.4.2 LOH and clinical stage

The most important prognostic factor in cervical cancer is clinical stage (Inoue *et al.*, 1984; Huttner *et al.*, 1998).

All previous studies of LOH in cervical carcinoma have included tumours of all stages and have provided some information regarding histopathological status and clinical features (Phyllis *et al.*, 1998). There was no significant correlation between the number of chromosomal arms exhibiting losses and any of the clinical of histopathological factors studied (Mitra *et al* 1994; Hampton *et al.*, 1994; Bethwaite *et al.*, 1995). Mitra *et al.* (1994) found that fractional allelic loss was not significantly associated with stage of the disease in cervical cancer. Wong *et al.* (1993) divided cervical cancers into early-stage (FIGO stage I-II) and the late-stage (FIGO stage III-IV) according to their clinical stages. The instance of LOH they observed in at least one of loci was 59% and 60% respectively. There was no significant difference in the incidence of LOH between early and late stage ($p > 0.05$. Chi-square test). But Konishi *et al.* (1993) observed that the region of 1p is frequently lost in neuroblastomas, colorectal carcinomas and phaeochromocytomas from patients with multiple endocrine

neoplasia type 2, suggesting that the same tumour suppressor gene was involved in the progression of different types of carcinomas (Konishi *et al.*, 1993).

We found that the incidence of LOH in advanced stage is significantly different from early stage in four loci namely D1S2829, D1S2757, D1S2647 and D1S2663 ($p < 0.05$). This suggests that the incidence of LOH remains different between early- and late-stage. This implies that LOH on these loci is related to progression of the tumours and has a higher incidence in the cancers of advanced stages. No significant difference was found between early- and late-stage carcinomas among other loci ($p > 0.05$), indicating that LOH on these loci does not correlate with any clinical stage and is unrelated to progression of the tumours.

5.4.3 LOH and pathologic grade

The LOH of D1S2878 is significantly related to high grade disease. There are no relationship between LOH and pathologic grades in other loci. It predicts that LOH has on relationship with pathologic grade at this locus.

5.4.4 LOH and clinical status

LOH on the loci of D1S2829, D1S2757, D1S2703, D1S2635, D1S2647, D1S2845, D1S461, D1S2663 and D1S76 was significantly associated with clinical status. Patient who had died of their disease or alive with evidence of disease had an elevated frequency and patient alive with no evident of disease has lower incidence. LOH on the remaining loci had no significance with clinical status.

Chapter Six

Conclusion

Cervical cancer remains one of the most common female malignancies worldwide. An increasing number of studies indicates that tumourigenesis is probably related to abnormal cell growth promoted by proto-oncogene and tumour suppressor genes.

Detection of LOH can provide a starting point for positional cloning of candidate tumor suppressor genes related to cervical cancer and can open new strategies for predicting the prognosis of cervical cancer. LOH of tumour samples was determined by comparing the intensities of bands obtained from tumour DNA with that from matched blood DNA. Only the case with 70% or more reduction of intensity in one band from tumour sample compared to that from blood sample was considered to be positive for LOH.

Tumour tissues and blood from 100 cases of cervical cancer were collected. Twenty nine microsatellite markers and two variable number of tandem repeats markers located on chromosome 1 were used in this study.

PCR is an artificial means of replicating a short DNA sequence, because of its speed and ease of use. we implemented [γ-32p]ATP which is highly sensitive and economic and only needs 0.5 μl (10 mCi/ml) for 50 reactions.

LOH was detected in 96 of 100 (96%) cases of cervical cancer. The number of tumours informative for each locus ranged from 40 % to 95%. LOH at two or more than two loci was detected in 88 of 95 (93%) informative cervical carcinomas, while 8 of 95 cases (8.4%) had LOH detected only at one locus, suggesting that chromosomal aberrations causing LOH on chromosome 1 are relatively common in cervical cancers. The most common site of LOH were 1p31 (44% of informative cases) and 1p36 (about 40% of informative cases), 1p36.3 (35%) and 1q25 (31%). The finding of 31-45% LOH at the loci D1S2829 (1p31), D1S76 (1p36-35), D1S80 (1p36-35), D1S2663 (1p36.3) and D1S2757 (1q25) strongly implicates these as the sites of one or more genes likely to play an important role in the origin or progression of cervical carcinoma which could be considered as the starting point for positional cloning of candidate tumour suppressor genes related to cervical cancer

Younger and older patients revealed no differences with LOH ($p > 0.05$). The incidence of LOH remains different between early- and late-stage on the loci D1S2829, D1S2757, D1S2647 and D1S2663 ($p < 0.05$). LOH at these loci maybe relate to the progression of cervical cancer.

For pathologic grades, only p value at locus D1S2878 was less than 0.05, revealing that on the locus LOH is associated with pathologic grade and progressive grade has a higher frequency of LOH. LOH at locus D1S2878 maybe relate to differentiation of cervical cancer cells.

LOH on the loci D1S2829, D1S2757, D1S2703, D1S2635, D1S2647, D1S2845, D1S461, D1S2663 and D1S76 was associated with clinical status and is significantly different ($p < 0.05$). Patients who had died of their disease or who alive with evidence of disease had elevated frequency of LOH at these loci. If these observations hold true in a large case study, screening for LOH at the loci in cervical cancer may be of use in identifying those patients who are at potentially higher risk for having poor outcome.

Chapter Seven

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